Poznan University of Medical Sciences
Poland

JMS Journal of Medical Science

previously Nowiny Lekarskie

Founded in 1889

2019
Vol. 88, No. 3

QUARTERLY

Indexed in:
Polish Medical Bibliography, Index Copernicus,
Ministry of Science and Higher Education, Ebsco, Google Scholar

eISSN 2353-9801
ISSN 2353-9798

www.jms.ump.edu.pl

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PUBLISHER
Poznan University of Medical Sciences

eISSN 2353-9801
ISSN 2353-9798

Publishing Manager: Grażyna Dromirecka
Technical Editor: Bartłomiej Wąsiel

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Ark. wyd. 6,9. Ark. druk. 8,3.
Zam. nr 103/20.

The Editorial Board kindly informs that since 2014 *Nowiny Lekarskie* has been renamed to *Journal of Medical Science*.

The renaming was caused by using English as the language of publications and by a wide range of other organisational changes. They were necessary to follow dynamic transformations on the publishing market. The Editors also wanted to improve the factual and publishing standard of the journal. We wish to assure our readers that we will continue the good tradition of *Nowiny Lekarskie*.

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ORIGINAL PAPER

DOI: https://doi.org/10.20883/jms.367

Minor Cannabinoids of *Cannabis sativa* L.

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ABSTRACT

Cannabinoids from *Cannabis sativa* L. play an important role as natural products in clinics. The major cannabinoids compromise tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA) and its decarboxylated analogs. In this review, we focus on often neglected minor cannabinoids and discuss biosynthetic and chemical degradation routes to other neglected cannabinoids in *Cannabis sativa* starting from THCA, CBDA and cannabichromenic acid (CBCA). Based on the literature, patents and scientific reports, essential routes for the chemical modification of cannabinoids are discussed to explain chemical diversity chemical conversion and degradation by UV light, as well as temperature and pH leading to the formation of structurally unusual cannabinoids *in planta* called as minor cannabinoids. Based on known bioorganic reaction schemes and organic chemistry, principles for minor cannabinoid formation like [2+2] cycloaddition, Markonov condensation, radical introduction, or aromatisation are discussed. Finally, the non-aqueous environment in *Cannabis sativa* trichomes is analysed to clarify their role of a miniaturised bioreactors the light-induced conversion in a non-aqueous enviroment. The overall objective is to bridge from metabolic profiling via cannabinomics to structural and chemical diversity that allows the definition of patterns with consequences also to pharmacology and plant breeding.

Keywords: *Cannabis sativa*, cannabinoids, tetrahydrocannabinol, cannabidiol, cannabichromene, cannabielsoin, cannabitriol, cannabinomics.

Biosynthesis of cannabinoids

Cannabinoids seem to be a unique class of natural products limited to *Cannabis sativa* L. only. In recent time, prenylated olivetolic acid derivates and other structurally related prenylated phenolics have also been identified in various genera and species like *Helichrysum umbraculigerum* Less. [1] or the liverwort *Radula marginata* TAYLOR [2, 3]. So far, the biosynthesis of cannabinoids towards to the biogenetically hybrid derived from the mevalonate and polyketide pathway as we understand today has only been detected in *Cannabis sativa* L., *Cannabis indica,* and *Cannabis ruderalis*, but recently as well in the New Zealand liverwort *Radula marginata* TAYLOR and *R. perittittonii* [3]. Without going into the details of molecular biology, genetics and spatial resolution of biosynthesis (**Figure 1**) [4], all committed biosynthetic enzymes involved in the formation of THCA-C5, CBDA-C5 and CBCA-C5 (**Figure 1**), cannabidiolic acid (**Figure 1**) and cannabichromenic acid (**Figure 1**) have a common precursor in cannbigerolic acid (CBGA-C5). and all conversion products have identical masses and differ only structurally.

Major structural differences refer to the alkyl chain length of the classic THCA-C5 and the short one in cannabivarin known as THCA-C3 (**Figure 2A**). Interestingly, no other biosynthetically relevant gene or enzyme has been found yet that may extend on the biosynthetic pathway from above-mentioned cannabinoids on. In contrast to more than 150 found so-called minor cannabinoids [5] this observation is provoking the question if these cannabinoids, detected at very low

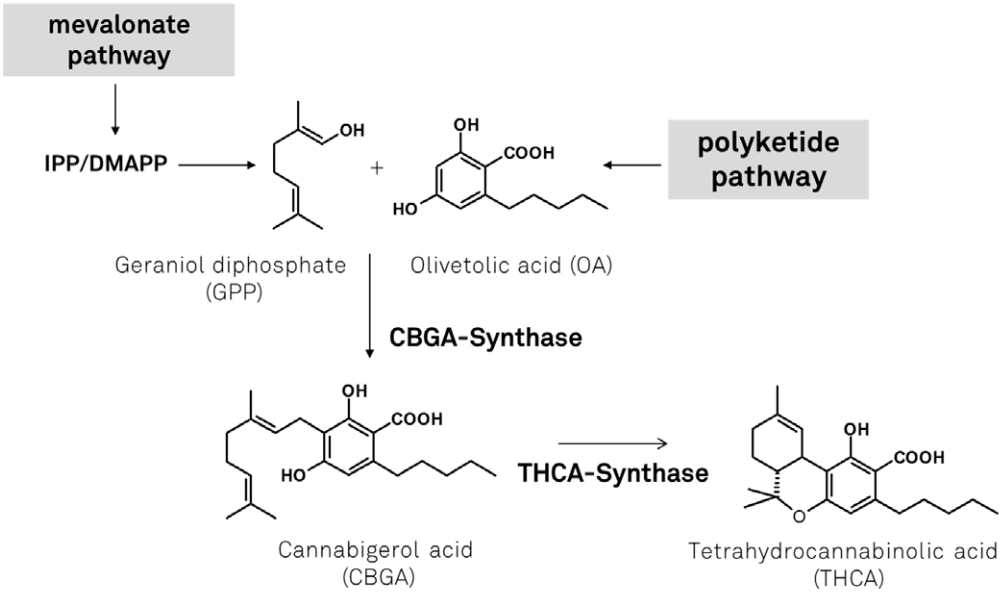
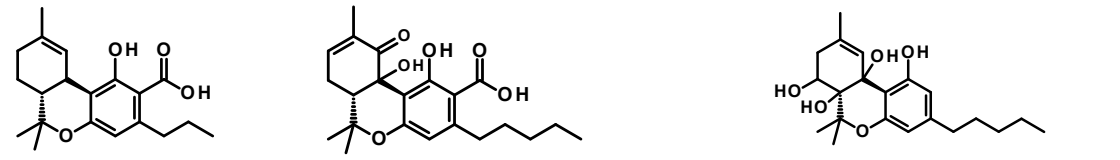


Figure 1. Basic concept of the tetrahydrocannabinolic acid biosynthesis in *C. sativa* L



A
Cannabivarin (THCA-C3)

B
Hydroxy-9-oxo-Δ^8-tetrahydrocannabinol

C
6a,7,10a-Trihydroxy-Δ^9-Tetrahydrocannabinol

Figure 2. Chemical structure of the most important cannabinoids with relevance to this contribution

concentrations *in planta*, are simply degradation products of chemical conversions induced by light, temperature or abiotic ecological factors.

Chemistry of cannabinoids

Structural diversity of cannabinoids is ruled by complex chemistry [6]. In principle, it can be distinguished between the polar carboxylated cannabinoids and the neutral lipophilic decarboxylated cannabinoids. Going deeper into structural aspects, all cannabinoids show a resorcinoyl core and mostly a side chain of three or five carbons. Flanking alkyl regions in the bicyclic region (menthyl-core) and the aliphatic chain are sensitive to oxidation [7]. Even the single double bond in position Δ^9 shows poor oxidative stability during storage of dried plant material. A closer look to the isoprenyl residue (C10) that has been formerly been the C-C attached GPP moety (**Figure 1**), can be found in four topological arrangements [5]:

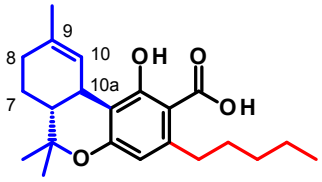
1. The simple connection via a Friedel-Crafts C-C alkylation as an electrophilic aromatic substitution towards CBG or in a second step reaction induced by UV light starting from CBCA-C5 to cannabicyclol type cannabinoids like CBL-C5 (**Figure 2**B). This closure of an additional carbon bond is by some authors considered to be an additional fourth topological arrangement [5].
2. Closure of the attached GPP-unit with the resorcinoyl hydroxyl group to chromenes as CBCA-C5.
3. Internal C-C reaction to a cyclohexane ring like in CBD followed by a nucleophilic reaction with one of the resorcinoyl hydroxyl groups resulting in hydrocannabinolics like THCA-C5

or canabielsoin-type cannabinoids (reaction with C_5-OH) like cannabielsoin E (CBE) (reaction with C_1-OH).

4. Aromatisation of the cyclohexane ring from CBDA-C5 or THCA-C5 after oxidation in the positions C_1, C_2, C_3, and C_5 or C_9, C_{10}, C_{10a}, C_7, C_8, respectively. Aromatisation gives CBN and CBDN-type cannabinoids.

Decarboxylation of cannabinoids is a spontaneous non-enzymatical process that is highly temperature dependent. Smoking will accelerate decarboxylation at high temperatures, but during storage decarboxylation with at an estimated rate of 5–10% a year is known in dried plant material at room temperature (20–25°C) as well [8].

Oxidation of the alkyl side chain is also reported in the literature. Here, oxidative processes are linked to microbial degradation [9]. The chain length can also be reduced by β-oxidation and the loss of a C2-unit. It is not anticipated that this is the major route to THCA-C1 (oricoids), because the presence of specific polyketide synthases (PKS) is responsible by biosynthesis for C1 but also C3 cannabinoids (viridinoids) [10].



Structural elements of cannabinoids explained for THCA-C5 with current numbering. Black: resorcinoyl-core; Blue: menthyl-core; Red: C5 isopentenyl-chain

Classification of cannabinoids

The chemical taxonomy and classification of cannabinoids are not ruled by the enzymatic or biosynthetic route. Here, we follow the standard classification as applied in organic chemistry based on the structure an arrangement of cyclic systems. In **Figure 3** a broad overview of the most important classes is depicted. Based on topological arrangements as discussed above, cannabidiolic acid (CBDA-C5) seems to be an important player. Mostly, CBDA-C5 is accepted as a cannabinoid on its own and catalysed by the enzyme CBGA-C5 synthase. But from the mode of catalysis and a theoretical carbocation in the CBD-like intermediate, we may have to consider CBDA-C5 cations as intermediates towards to THCA-C5 (**Figure 4**). This has the implication, that CBDA-C5

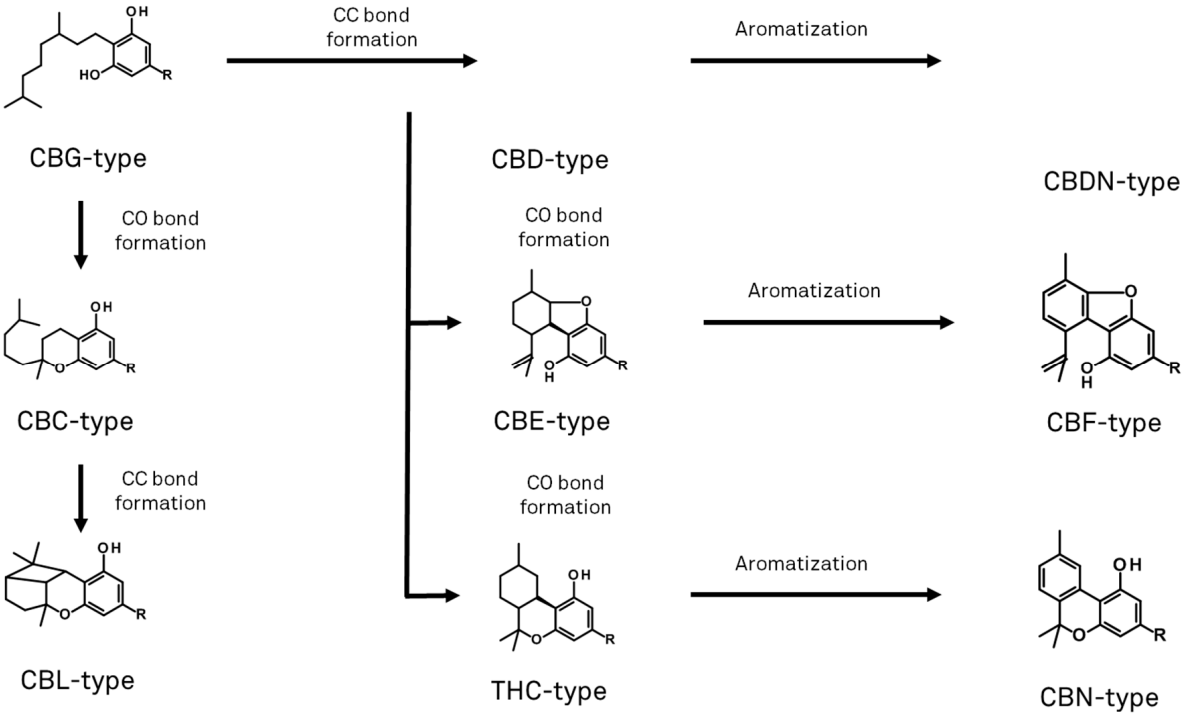


Figure 3. Structural diversity of cannabinoids based on biosynthetic and chemical conversions

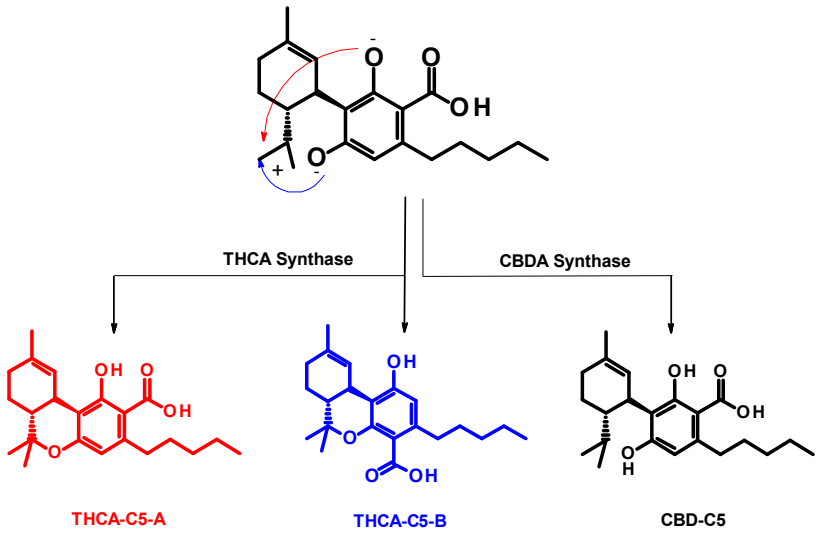


Figure 4. Role of CBD in the catalytic conversion process

is rather unstable and can be activated by appropriate conditions. This may explain, why CBD-C5 can be converted to THC-C5 at harsh conditions like pH 1.

Although we know that the homology between CBDA Synthase and THCA Synthase is 88% [11], we need to address the question how the carbocation is relocated in the intermediate activated CBDA-C5 intermediate and how it is stabilised in the active center of the enzymes. This question cannot be answered today, but it is of high interest under the proven assumption that each one of the three enzymes (CBDA Synthase, THCA Synthase, and CBC Synthase) can convert in principle CBGA-C5 to all three products THCA-C5, CBDA-C5 and CBCA-C5. If so, we may have an answer to why the fiber hemp the plant still produces THCA-C5 at concentrations below 0.3% and we never get a *Cannabis* plant that is free of THCA-C5.

If we consider this basic question of carbocation relocation and structural diversity, we may have a first approach towards a better understanding for classification based on a hierarchy of chemical reactions. First, C-C coupling is based on an enzymatic reaction by a prenyltransferase, second, carbocation formation and another C-C coupling to a bicyclic system, third, additional cyclisation based on nucleophilic reaction with hydroxyl groups or electrophilic addition with a carbocation to three-membered aliphatic, aromatic or heteroaromatic ring systems.

Formation of minor cannabinoids

Δ8/Δ10- THC

In *Cannabis sativa* L. the position of the double bond is due to the localisation in the GPP and its addition to the aromatic ring at the position C9. Commonly this is indicative for the nomenclature of Δ^9 and represents 99% of THCA-C5 and THC-C5 in the plant. Other positions of the double bond in the positions C8 and C10 are considered as artifacts introduced by acids or oxidatively promoted a shift of the endocyclic double bond. Main reason, as observed in aged plant material, is oxidation under UV light influence or in the lab at drastic acidic (pH1) conditions, as observed for CBD-C5 cultivations also yielding Δ^8/Δ^{10}- THC (**Figure 5**). For CBD-C5 it must be mentioned, that UV light exposure is irrelevant for the conversion to THC as documented by unpublished experiments of the author.

Cannabinol (CBN-C5)

Presence of aromatised THC known as cannabinol (CBN-C5) (12,13) is mostly detectable in aged *Cannabis sativa* L. If cannabis material or the pure isolated THC is exposed to UV light or sun light for an extended amount of time, the menthyl-ring system will be oxidised as suggested in **Figure 6**. Oxidation followed by dehydration resulting additional double bonds is the main road to full aromatisation. The order of stepwise induced double bonds is not clear, but it can be

assumed that the first oxygen attack is at position C10a as energetically most favored followed by a C6 attack. In parallel, other various intermediates like C6-C9 overbridged peroxide derivates as instable intermediates are discussed as well. A C9-C10 epoxide was detected, but seems unlikely to contribute to the aromatisation process [14]. In general, oxidation in the menthyl ring is often observed in plants and represents the most prominent way of oxidation in this kingdom. For instance, double oxidation like in 10-Hydroxy-9-oxo-Δ^8-tetrahydrocannabinol (**Figure 2B**), dou-

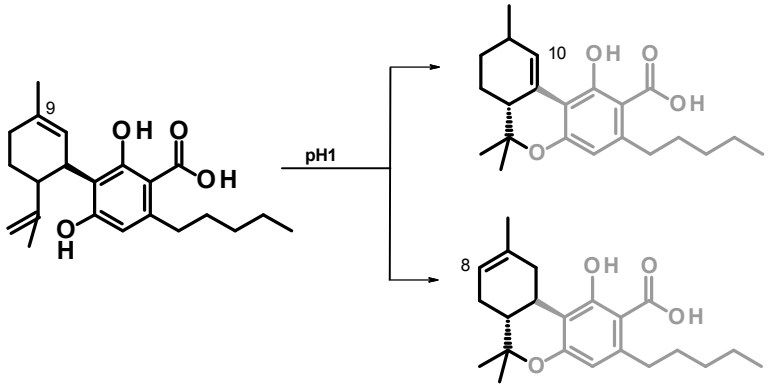


Figure 5. Chemical conversion of CBD-C5 to Δ^8/Δ^{10}- THC at pH 1

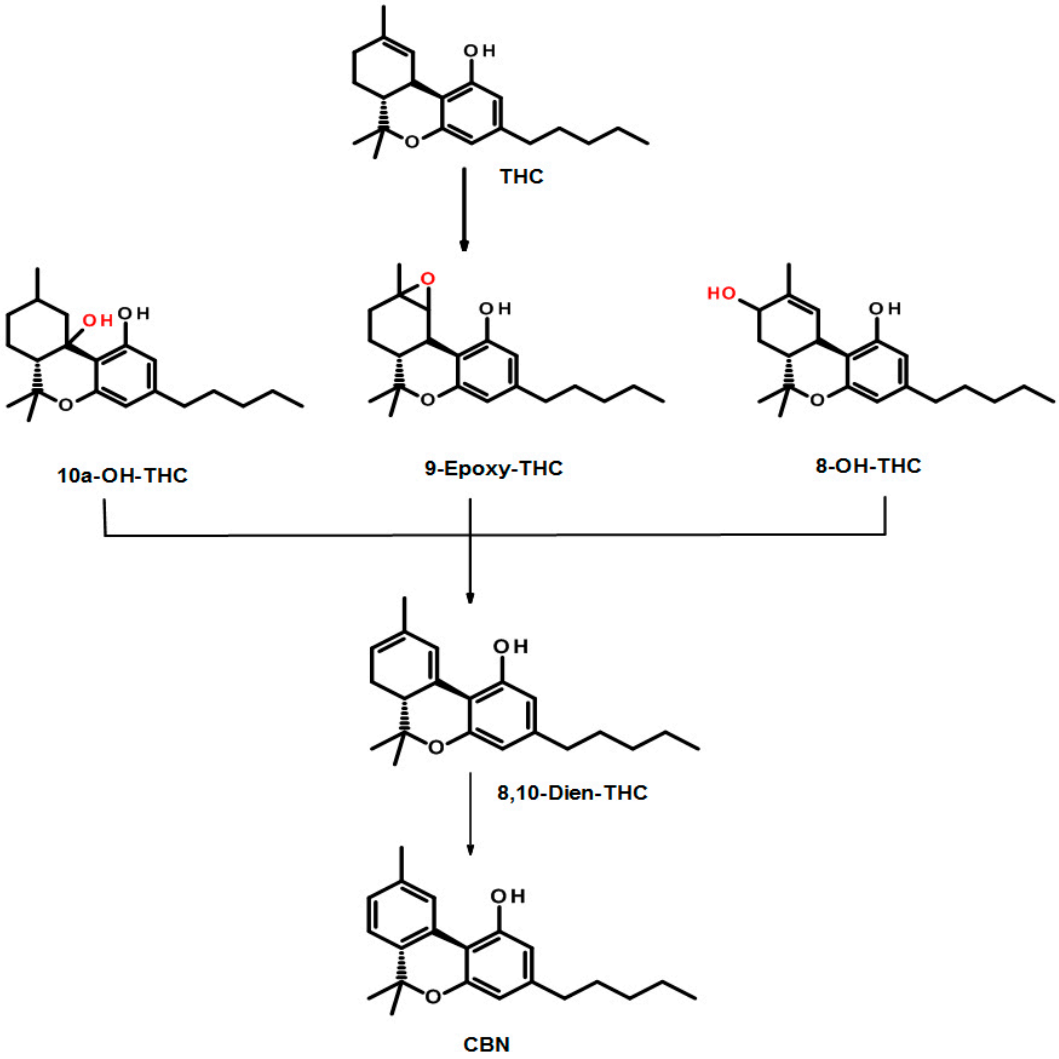


Figure 6. Proposed oxidation of THC under UV light towards CBN-C5

ble oxidation to Δ^9-trans-Tetrahydrocannabinol glycol (Cannabiripsol) [15], triple oxidation to 6a,7,10a-Trihydroxy-Δ^9-Tetrahydrocannabinol (**Figure 2C**) or simple 10 α/β-oxidation are typical reactions in plants. It is of interest, that microsomal oxidation by human liver cytochromes and by certain microorganisms take place in the exocyclic methyl group at C11 or in the alkyl chain [9]. It must be mentioned that CBDN-C5 as aromatised product from CBD-C5 follows the same chemical routes as described above. Here, we will not go into detail again, but the steps of oxidation and the same photochemistry can be applied as well.

In recent publications (-)-trans-Cannabitriol (CBT-C5) is mentioned as a minor cannabinoid on its own [5, 16]. Here, we do not consider CBT-C5 as a minor cannabinoid of elevated importance for the plant. We regard CBT-C5 to be a highly oxidised intermediate. Doubtful is the identification of ethoxy structures which are more likely to be artifacts by ethanol extraction [16].

Cannabichromene (CBC-C5) and Cannabicyclol (CBL-C5)

To our knowledge, the acid form of cannabichromene (**Figure 2C**) [17] is the last out of the three major compounds that are biosynthesised by an enzymatic reaction (CBCA synthase) [18]. Although it is not considered a minor cannabinoid in *sensu stricto*, CBCA accumulates in concentrations of around 0.2–1% [17] in various *Cannabis sativa* L. strains, which is significantly lower than their respective THCA-C5 and CBDA-C5 analogs. CBCA-C5 and CBC-C5 is racemic and can also easily be made by synthesis [19].

Cannabicyclol (CBL-C5) is a conversion product of CBC-C5 under the influence of light (**Figure 7**) [19]. The chemical mechanism can be explained as followed: After UV irradiation, a Lewis acid-catalysed intramolecular ene reaction between two partners occurs. For CBC, one ene in the chromene ring of CBC-C5 reacts with remaining one in the former GPP rest to give a carbocyclic four-membered ring in CBL-C5 [21]. It must be mentioned that in cannabis CBL-C3 known as CBLV-C3 and the carboxylated CBCA-C3 are also detected.

Cannabielsoin (CBE-C5)

This cannabinoid type is characterised by a furan ring as a result of a formal intramolecular opening of the 9-epoxide THC (**Figure 6**). It is not clear if the CBE-type compounds are natural products or artifacts in the isolation process. By mechanism, a radical initiation is also likely and shown for the conversion of CBD in plant cell cultures [22]. The absolute stereochemistry is 5aS,6S,9R,9aR [19]. *In planta*, carboxylated metabolites like cannabielsoic acid A, B, C1-C3 are known. Both occur with a C5 or a C3 aliphatic chain. Cannabielsoin is also detected after administration of CBD-C5 to humans and guinea pigs [23, 24]. The mode of liver metabolisation is unclear, but radical formation of a reactive intermediate by a cytochrome and coupling with the C1 hydroxyl group is likely.

Discussion

Minor cannabinoids by the definition of low concentrated cannabinoids in *Cannabis sativa* L. are an exciting group of natural products. We learned that it is likely that most of the minor cannabinoids are unlikely to be biosynthesised by an enzymatic biotransformation that rather photochemical conversion or other reactions are important as we know from organic chemistry (**Table 1**). Chemical diversity in cannabinoid species is a consequence of the catalytic promiscuity of THCAS, CBDAS and CBCAS at the beginning of biosynthesis followed by a series of non-rational oxidation and C-C coupling steps in a non-stringent order. If we consider photochemical reactions, these will primarly occur in the trichomes as main biosynthetic organ. Trichomes are append-

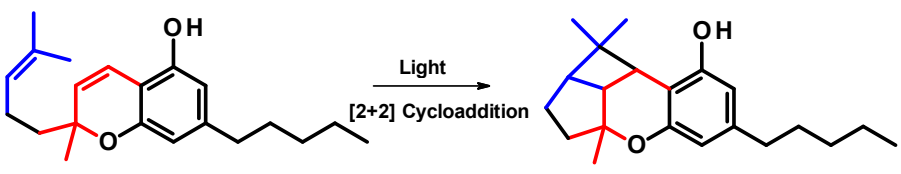


Figure 7. Proposed [2+2] cycloaddition from CBC-C5 (left) toward CBL-C5 (right)

Table. 1. Chemical organic reactions of cannabinoids

Type of reaction	Type of reaction and position in the cannabinoid structure	Example for cannabinoid conversion
Makonovikov-type protonation	Endocyclic double bond and tertiary C9	cannabicitran
Radical reaction	Epoxidation	cannabielsoin
[2+2] Cycloaddition	Ene coupling	cannbicyclol
Oxidation epoxidation	Oxidation of non-aromatic double bond	cannabitriol, cannabidiol glycol and more
Dimerization	Radical induced aromatic coupling	
Steric rearragenments	Wagner-Meerwein relocation	cannabicyclol

ages or aerial epidermal surface hairs on plants with two main features. First, trichomes consist of secretory cells and second, secreted exudate is stored in an extracellular cavity also called essential oil container. The localisation of the biosynthetic proteins for CBGA-C5 and THCA-C5 is still unclear. Most authors assume membrane bound (CBGAS) and cytosolic (THCAS) localisation of both enzymes. THCA and CBDA or decarboxylated analogs are secreted or diffuse into the essential oil container. But in recent reports by Mahlberg et al. 1992 [25] reported THCAS to be detectable outside of the cell wall of the glandular cell, sticked in the membrane and catalysing at non-aqueous conditions. This was confirmed by Rodziewicz and Kayser, 2019 [26] who clearly documented by proteome analysis the presence of THCAS in the essential oil.

Trichome may play in the physical way an important role as well. Specific physical properties may also contribute to the unique reaction conditions inside of trichomes. A near perfect spherical shape, observable in the lower micrometer range, strongly promotes light refraction and focusing through lensing effects in a bulb filled with essential oil. These exact conditions of extreme sphericity in shape and structure are found in the globular essential oil reservoirs of trichomes. An increase of temperature up to 50°C in direct sunlight is likely and accelerates radical formation for the discussed C-C binding. Other reactions as well as mentioned in **Table 1** are temperature dependent and may promote conversion to minor cannabinoids. These physical conditions for chemical conversion in trichomes have not even raised so far and yet may play an important tole for the understanding of non- aqueous organic chemistry in these biobased reactors.

We have not discussed minor cannabinoids with a C3 or C1 alkyl chain here. In total, the number of minor cannabinoids is estimated to compromise at least 150 compounds. Many of these compounds seems to be artifacts of downstream processes and some like quinones are only stable after acetylation or O-methylation. In addition, dimerisation is known although not considered as a biogenic process and isolated structures are known in very low concentrations only. Dimerisation is anticipated as a radial-initiated organic reaction of the resorcinyl-core. It is interesting that plants mostly modify the pattern by oxidation in the aromatic and menthyl-cores of cannabinoids, while mammalians oxidise C11 methyl group and microorganisms mostly impact the alkyl chain. We do not know about or just have overlooked this diversity and it is worth it to look closer with sophisticated analytical mass spectroscopy.

The biological role and function of minor cannabinoids for the plant is not known. On a closer look, the postulate that all natural products must have a specific benefit for the simple sake of energy optimised plant physiology, may not be true in this case. The previously discussed extracellular non-regulated synthesis being out of the control of the plant is special. Nevertheless, the usability in human medicine seems most obvious and in the future minor cannabinoids will definitively be in the interest of pharmaceutical companies.

Conclusion

After the legislation of *Cannabis sativa* L. for medical use, research and development is exploding (**Figure 8**) and still huge amounts of investments take place to push pharmacological studies. After only 5 years of legalised research in the USA, Israel, Canada and Germany, we merely can see the tip of the iceberg and more research in the interest of both the patient and consumer is welcome and needed. If we can see today a great interest in THC-C5 and CBD-C5 by industry and academia, it is more than likely that companies and scien-

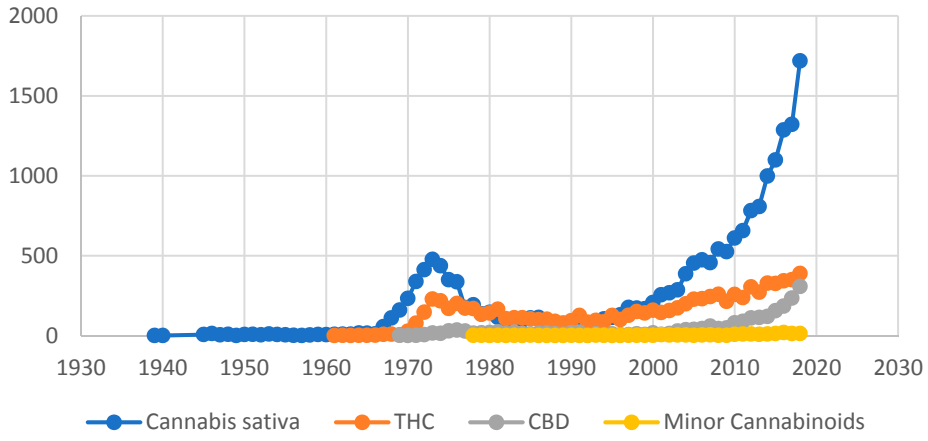


Figure 8. Number of publications about cannabis, THC, CBD and minor cannabinoids from 1939–2018

tists will focus in the near future on the unexplored world of minor cannabinoids in the near future. A limiting factor for extensive research is the low availability and high price for minor cannabinoids. Extraction and purification of these compounds from cannabis extracts is challenging due to extreme low yield and low stability during downstreaming. Alternative strategies like organic synthesis or biotechnological production will be attractive and competitive to plant-based production in the future. The chemical diversity of cannabinoids is high and still unexplored, but we know that the biosynthetic impact by enzymatic catalysis on diversity was overestimated and photochemical conversion in a non-aqueous trichome "bioreactor" is an accepted hypothesis to explain pattern and occurrence. Beyond the horizon of CBD-C5 and THC-C5 dominated research and business today, we can expect new disruptive findings to establish minor cannabinoids in pharmacology and clinics, why the story of the one-billion-dollar plant is not told yet.

Acknowledgements

Conflict of interest statement
The authors have no conflict of interest with any commercial enterprise.

Funding sources
There are no sources of funding to declare.

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Acceptance for editing: 2019-07-09
Acceptance for publication: 2019-09-30

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ORIGINAL PAPER

DOI: https://doi.org/10.20883/jms.357

Nickel-free environment — Dreams vs. Reality. Everyday utilities as a source of nickel and cobalt for patients sensitized to these metals

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ABSTRACT

Introduction. Frequent occurrence of elevated nickel levels in everyday items explains why allergic contact dermatitis to nickel is the most common in the general population. In Northern America and Europe, 20% of the general population suffers from contact dermatitis while 8.6% of patients suffering from contact dermatitis are allergic to nickel.
Material and Methods. A group of 25 patients (24 females and 1 male) sensitized to nickel and cobalt on the basis of patch testing was analyzed during a 2-year-long period in Department of Dermatology Poznań University of Medical Sciences. Contact allergy to nickel and cobalt was confirmed with the positive result of patch test, conducted with the Polish Standard Series of chemotechnique.
Results. An excessive nickel release was detected in over a quarter of the tested items, respectively in 7.5% of jewellery, 57.89% of clothing accessories, 56.89% of other utility goods, such as keys, telephones or stationery. Cobalt excessive release was found in 7.3% of tested items, respectively in none of jewellery and kitchen accessories, 25% of clothing accessories, 12.5% of other utility goods (keys, pens, pendants).
Conclusions. In general, everyday-use items are not nickel-free and more legislation steps are necessary to provide it and prevent initial sensitization in future generations. Several articles of every-day use release nickel and cobalt above migration limits.

Keywords: nickel allergy, cobalt allergy, Nickel Directive.

Introduction

Frequent occurrence of elevated nickel levels in everyday items explains why allergic contact dermatitis to nickel is the most common in the general population. In Northern America and Europe, 20% of general population suffers from contact dermatitis while 8.6% of patients suffering from contact dermatitis are allergic to nickel. Sensitization to cobalt is described as one of the most common allergies to metals [1]. Due to a high prevalence of its occurrence, it is a health concern in the European Union [2].

The initial outbreak of sensitization to nickel is dated on early '70s (popularization of buttons and zippers of blue jeans) [3] followed by the development of ear-piercing trend in the '80s [4]. To reduce the prevalence of nickel sensitization in younger generations the Nickel Directive (currently known as nickel restriction) was estab-

lished in Denmark in 1994 [5]. In Poland, the regulation came in full force in 2005, after joining the European Union.

Aim

According to our data research from several publications [6, 7], decrease of the prevalence of nickel allergy in younger girls in Western European countries is undeniable. As the aim of our study we took an estimation of possibility to eliminate metal items containing nickel or cobalt from the environment of sensitive patients.

The primary objective of the study was to assess whether the patients sensitive to nickel or cobalt were able to eliminate all metal items containing these elements from their daily environment, furthermore, to determine objects of everyday use with a high concentration of nickel to help patients in removing sources of sensitization. We also aimed to educate patients in regards of skin exposure avoidance and allergic reactions to nickel-containing items.

Material and Methods

A group of 25 patients (24 females and 1 male) sensitized to nickel and cobalt on the basis of patch testing was analyzed during a 2-year-long period in Department of Dermatology Poznan University of Medical Sciences. Patients were included in the study if they had a positive reaction to patch test to nickel sulfate 5% or cobalt sulfate 5%. Contact allergy to nickel and cobalt was confirmed with the positive result of patch test conducted with the Polish Standard Series of chemotechnique. An examination of each patient consisted of extensive interview and questionnaire.

Structure of the questionnaire

The purpose of the questionnaire was to obtain an information on patients' demographic data, age, ear-piercing, chronic conditions, medicine intake and family medical history, including allergies among family members. Moreover, we asked about the presence and characteristics of patients' allergies — duration of symptoms, time of the diagnosis, location and appearance of first skin lesions and current lesions.

Subsequently, the patients answered four questions regarding ways of elimination of sensitizing objects:

What utility goods have you eliminated from your environment after the diagnosis of allergy?

Have you taken measures to reduce skin contact to metal items, such as using plastic substitutes or painting the surface of metal items with colourless varnish?

How do you assess the effects of eliminating the above-mentioned utility goods on the course of your allergy?

Is there any metal object essential in your everyday life/ workplace which you cannot eliminate despite the allergy?

Applied tests

The patients were asked to bring personal items or utility goods for examination. The items were supposed to be the source of nickel or cobalt allergy. A total of 229 metal items including jewellery, clothing accessories and other objects of everyday use were tested with the Chemo Nickel Test to assess nickel release. A total of 55 articles were tested with Chemo Cobalt Test to detect cobalt release. According to the producers: The Chemo Nickel Test detects free nickel down to a limit of 10 ppm (parts/million). Sensitivity threshold of most nickel allergic patients is above 11 ppm. Some strongly sensitized patients will however still react to objects releasing amounts below the threshold of the test. Chemo Nickel Test TM consists of an ammoniacal solution of Dimethylglyoxime (DMG) for the detection of nickel in various metallic objects. To perform the detection we put a few drops of the reagent solution onto the cotton tip to moisten it, and then rubbed the metal surface of the suspected object intensively for up to 1 minute. If the cotton tip changes colors into reddish-pink, this indicates the presence of nickel.

Results

A total of 58 patients who presented with a positive result in Patch Testing declared participation in the study. Finally, the analyzed group consisted of 25 patients (participation rate 43.1%), 33 patients did not come to scheduled appointment.

75% of patients reported eliminating of some metal utility goods from surrounding environ-

ment (for example metal watches, jewellery, pens). 54.17% of participants took measures to reduce skin contact with metal items (mainly by exchanging metal objects, such as cutlery, to plastic ones). Among the participants who reduced skin contact or the use of metal objects, 66.67% noticed an improvement in allergy symptoms afterwards, while 16.67% found it hard to evaluate the change and 16.67% did not benefit from the elimination. 75% of the participants stated they were not able to eliminate some essential metal objects despite confirmed allergy, mostly keys, pots and cutlery.

An excessive nickel release was detected in over a quarter of the tested items (**Table 1**), respectively in 7.5% of jewellery (6 out of 80 items) (**Table 2**), 57.89% of clothing accessories (11 out of 19 items) (**Table 3**), 56.89% of other utility goods, such as keys, telephones or stationery (49 out of 86 items) (**Table 5**). None of the tested kitchen accessories (41 items, including cutlery, frying pans, pots and salt cellars) showed an excessive nickel release (**Table 4**).

In jewellery, the subcategory with the highest rate in detecting nickel was bracelets and watches — 21.05% (4 out of 19 items). We did not identify nickel in any earring of 24 pairs we examined. (**Table 2**) In clothing accessories, the subcategory with the highest rate in detecting nickel were belts — 83.33% (5 of 6 belts), followed by zippers and buttons of jeans- 55.56% (5 out of 9) (**Table 3**). In the category of other utility goods, the highest rate in positive nickel detection relates to keys — 78.26% (36 out of 46 keys), followed by stationery and key accessories — 40.63% (13 out of 32 items). We did not identify nickel in any examined telephone or telephone accessory (8 items) (**Table 5**).

Cobalt excessive release was found in 7.3% of tested items (4 out of 55 items), respectively in none of jewellery (23 items) and kitchen accessories (4 items), 25% of clothing accessories

Table 1. Detection of nickel in tested utility goods — results

Number of tested objects, (n)	Chemo nickel test — positive, % (n)	Chemo nickel test — non-diagnostic, % (n)
229	27.51% (63)	27.95% (64)

Table 2. Detection of nickel in tested jewellery — results

Type of jewellery	Chemo nickel test — positive % (n)	Chemo nickel test — non-diagnostic % (n)	Number of tested objects (n)
Jewellery in total	7.5% (6)	25% (20)	80
Earrings	0	25% (6)	24
Necklaces	6.25% (1)	37.5% (6)	16
Rings	5% (1)	10% (2)	20
Watches, bracelets	21.05% (4)	31.58% (6)	19
Other	0	0	1

Table 3. Detection of nickel in tested clothing accessories — results

Type of clothing accessories	Chemo nickel test — positive % (n)	Chemo nickel test — non-diagnostic % (n)	Number of tested objects (n)
Clothing accessorries in total	57.89% (11)	15.79% (3)	19
Belts	83.33% (5)	0	6
Buttons of jeans	50% (2)	25% (1)	4
Glasses	25% (1)	0	4
Other, e.g. bag clasps, zippers	60% (3)	40% (2)	5

Table 4. Detection of nickel in tested kitchen accessories — results

Type of kitchen accessories	Chemo nickel test — positive % (n)	Chemo nickel test — non-diagnostic % (n)	Number of tested objects (n)
Kitchen accessories in total	0	43.90% (18)	41
Cutlery	0	46.15% (12)	26
Dishes, pots	0	40% (4)	10
Other, e.g. salt cellars, graters	0	40% (2)	5

(1 out of 4 items — a button of jeans), 12.5% of other utility goods (keys, pens, pendants — 3 out of 24 items). Among jewellery only one necklace released cobalt. We tested 13 keys and only two of them released cobalt extensively (**Table 6**).

Out of 25 research study participants, 96% were females. Apart from allergy to nickel and cobalt, almost 30% of patients were allergic to other substances, such as palladium, and cosmetic allergens (**Table 7**).

both sexes is comparable, no difference in prevalence of sensitization to nickel between genders has been observed [9].

The number of piercings and the incidence rate of nickel allergy are strongly associated among both genders, the association being higher in males [10, 11].

The highest rate in nickel detecting in every day utilities was observed in keys, however, according to the defi nition of prolonged contact

Table 5. Detection of nickel in other tested utility goods — results

Type of utility goods	Chemo nickel test — positive, % (n)	Chemo nickel test — non-diagnostic, % (n)	Number of tested objects, (n)
Utility goods in total	56.98% (49)	17.44% (15)	86
Keys	78.26% (36)	17.39% (8)	46
Key sheaths, key rings	40% (2)	60% (3)	5
Telephones, telephone accessories	0	25% (2)	8
Other, e.g. stationery, clips, pens	40.74% (11)	7.41% (2)	27

Table 6. Detection of cobalt in tested utility goods — results

Type of utility goods	Chemo cobalt test — positive, % (n)	Chemo nickel test — non-diagnostic, % (n)	Number of tested objects, (n)
Utility goods in total	7.27% (4)	7.27% (4)	55
Jewellery	0	4.35% (1)	23
Clothing accessories	25% (1)	0	4
Kitchen accessories	0	0	4
Other	12.5% (3)	12.5% (3)	24

Table 7. Characteristics of research study participants

Property	Characteristics of research study participants, (n)
Gender	95% Females (24), 5% males (1)
Average age	47 Years
Ear-piercing	68% (17)
Monovalent allergy to nickel	72% (18)
Allergy to nickel and cobalt	24% (6)
Other allergies	28% (7)

Discussion

Contact dermatitis is manifested by an itchy rash appearing a few hours after skin contact with the allergen [8], so it is essential to avoid skin contact with nickel or cobalt containing metals.

The disproportion between genders observed in the study (24 female patients:1 male) is most likely associated to the difference in exposure to jewellery between men and women in our culture.

On the contrary — in Nigeria, where men and women wear jewellery equally and piercing rate in

with the skin it may be rather unlikely to cause allergic contact dermatitis.

Moreover, metal clothing items were identified as a significant source of nickel in our study. It was released by 57.89% of them, including belts, buttons, zippers, etc. Cheong et al. support our findings in their study; they revealed presence of nickel in 76.3% of metal clothing items from Korean markets. They also examined jewellery as a potential source of nickel, with positive results in 42.3% of them. In our study only 7.5% of jewellery was associated with nickel release. The difference may be associated with less effective

regulations regarding the release of nickel from metal products in Korea. Cobalt release was presented in 7.27% of all items in our study and in 6% of all items in Korean study. In both studies, all items releasing cobalt were also positive in the nickel releasing test [12].

According to the meeting of Nickel Institute Association in Brussels in 2017 the main reason of nickel sensitization persistence in European society are: sensitization before regulation was indicated (older individuals), the regulation might be too weak (necessity to decrease migration limits), violation of the regulation, lack of control by authorities, new items causing nickel allergy — e.g. laptops, phones and other sources not covered by regulation: toys, medical devices, coins, occupational.

The legislation stipulates that items intended to come into direct and prolonged contact with the skin are not allowed to release nickel above "migration limits", which is more than 0.5 ug/cm^2/week and 0.2 ug/cm^2/week for items intended to be inserted into human body (pierced ears or other body parts).

In 2014 European Chemical Agency defined "prolonged contact with the skin" as potentially more than 10 minutes on three or more occasions within two weeks and more than 30 minutes on one or more occasions within two weeks [13].

The implementation of the EU Nickel Directive caused a decrease in sensitization rate to nickel, especially among young women [14]. The median age of women participating in our study was 48 years, which may confirm that the nickel registration is sufficient because there was only one female participant under 25 years (4.17%). Other study shows that women ear pierced after 1990 were less likely to develop nickel allergy and dermatitis than women pierced before the introduction of regulations [15]. However, the incidence rate is still high and amounts to 8–18% of the general population [16] and 10% of young women being nickel allergic [15] (12.3% of 15-year-old females in a Polish study [17]). Higher nickel allergy prevalence rate is observed in southern than in northern EU countries [14].

The weakness of the present study is the human factor. Our aim was to assess whether patients sensitive to nickel or cobalt were able to eliminate metal items containing that elements from their environment, although some patients did not show any motivation in this process. For example, for some participants it was hard to part ways with favorite trousers despite the rash appearing on stomach near zipper area appearing few hours after skin contact. Some of the participants claimed to forget to put layers of nail polish on earrings which used to cause dermatitis because it was not important enough for them to prevent skin dermatitis. Other weakness of our study is a number of tested patients, which was relatively small.

Conclusions

Our investigation shows that patients who are strongly motivated are able to remove most sources of nickel or cobalt contact dermatitis from their environment. In general, every day-use items are not nickel-free and more legislation steps are necessary to provide it and prevent initial sensitization in future generations. Several articles of every-day use release nickel above migration limits. More common items need to be covered by the nickel regulations, especially keys, zippers and pedants. Some subgroups of every day use items, for example, cooking tools and earnings were found by us as nickel-free which is satisfactory and brings hope for sensitive patients to extend that profile to all items in the future.

Acknowledgements

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
There are no sources of funding to declare.

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Acceptance for editing: 2019-07-23
Acceptance for publication: 2019-09-15

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ORIGINAL PAPER

DOI: https://doi.org/10.20883/jms.323

The role of intra- and interpersonal relations in the process of diagnosis and treatment

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ABSTRACT

Introduction. There is an increasing tendency to adopt biopsychosocial approach to teaching how to care for patients. Participation in Balint's groups is used to train students in communication and building relations with patients.
Aim. To identify positive and negative aspects of participation in Balint's groups, which are a part of compulsory training for students.
Material and Methods. 70 medical students, who took part in the study, filled in a questionnaire specifically developed for students participating in Balint's group. The questionnaire consisted of three open questions. The answers were collected and then analyzed by way of qualitative analysis of text and factorial analysis
Results. The results suggest that Balint's method can be difficult for medical students because they have not practiced building therapeutic relations with patients. Nevertheless, most students benefit from training in terms of personal development, awareness of mechanisms influencing patients — doctor communication and satisfaction with participation in classes.
Conclusion. Balint's Workshops is a useful method of teaching which influences medical student's self — reflection because they become aware of the necessity of personality development.

Keywords: Balint's groups, interpersonal relations, treatment.

Introduction

Medical doctors have to work on their competence and ability to make right decisions although at the same time they have to cut up on examination and treatment costs. Participation in Balint workshops may let them develop their professional skills and increase their job satisfaction. This method was created by psychiatrist Michael Balint in 1950 in order to support doctors working with psychosomatic patients. Balint believed that patient — physician relation could have an ability to heal by nature but only if the doctor had necessary interpersonal competence to see patient's psychosocial situation. Since expertise in diagnostics and treatment methods is necessary but not sufficient to good medical practice, Balint designed a system of training in groups which was destined primarily for medical doctors. He was mainly interested in building relation between a patient and a doctor, the emotions and attitudes it evoked as well as its consequences [1].

Several years' experience in Balint workshop method of training have shown that it is difficult

to translate this method into clinical and theoretical framework because it may mean something else to everyone. In the beginning patient — doctor relation may be disturbing for students because they find it senseless to make in — depth analyses of this interaction, they try to treat it in a humorous way but then they discover the real value and true meaning of participation in Balint's workshops. Balint believed that building interpersonal skills is one of the priorities of academic medical training because in his opinion physician's personality, mood and reactions remain an important diagnostic and therapeutic tool [1].

In order to improve his/her communication with patients a doctor must recognize his/her behavioral model, a pattern of typical reactions which may influence his /her relation with a patient.

Group work is a way not only to acquire knowledge but also to make participants aware of their own influence on patient — doctor relation and their responsibility for this interaction. The participant may also gain the ability to dissociate oneself from the emotions which may be imposed by some patients, reduce the tension and cope with his/her own or patient's aggression, particularly with psychosomatic patients. Psychosomatic disorders are poorly understood thought to be a "blind spot of medicine [2]. These conditions are often neglected by psychiatrists although they are closely related to functional disorders. To add, somatization disorders create economic burden because they lead to long-term treatment and make additional medical tests necessary. The diagnosis of psychosomatic or somatization disorders could be difficult both for the doctor and for the patient, who may become frustrated [3]. Psychosomatic patients may present with general health problems or illnesses. Psychosomatization in itself remains an illness process, which could be understood in a number of ways e.g. as a reaction to stress. It seems to be common in medicine but not all psychosomatic patients manifest somatoform disorders. A lot of patients suffer from transient disorders or may somatize due to significant stress. Additionally, factors which interfere with patient's view of the world or their self-image may cause anxiety or fear. Humiliation, a sense of limited freedom, loneliness, losing job, financial problems, death or loss of a loved one, feeling unaccepted, guilty feelings, misery, chronic despondency or low self — esteem trigger permanent internal conflicts, which may in turn be manifested as psychosomatic disorders, intellectual deterioration, decreased resilience or negative, pessimistic attitude to one's existence. Psychosomatic illnesses could be caused by inadequate processing of negative emotions such as animosity, aggression or depression [4].

Here, it is important to note that treatment of psychosomatic patients may be challenging for young doctors, who can react to them with fear or defense, believe the patient is malingering or think the symptoms are patient's fault [5]. They often believe their reactions to these patients such as impatience, embarrassment, anger, helplessness, sadness or surprise are patient's fault. To add, they often use "militant" vocabulary for example they believe these patients should be "harnessed", "mastered", "pacified", "humbled", "put in his/her place", referred for an unpleasant examination, sent to a psychologist/psychiatrist to punish them or sign off if they do not appreciate the treatment they receive.

Material and Methods

The study involved seventy 3rd year medical students of Poznan University of Medical Sciences who participated in three-day Balint group meetings. The training consisted of three meetings. The initial theoretical meeting was devoted to communication with a psychosomatic patient and the mechanism of transference and countertransference. The next two meetings included practical workshops in groups of 10–14 students which focused on student's relation with patients. Their individual case reports were based on students' experience achieved during obligatory summer practices in nursing and in a general practitioner's office. During each three — hour class students were able to discuss two or three case reports on average. Group meetings, which were tutored by certified Balint group leaders, focused on interaction between a medical student and a patient. To reduce students' initial tension and anxiety tutors stressed that a Balint's group is not therapeutic in character and its goals are not related to solving personal problems but to psychological relation between a patient and a doctor without in — depth analysis of participants' personality. The meetings do not involve moral or professional evaluations,

diagnosis or "good advice". moreover, each participant of a group has to formulate his/her own conclusions [1]. To estimate the effectiveness of group work a questionnaire for Balint group participants was used. The questionnaire focused on:

1. Each student's knowledge about group work (e.g. functioning of social groups, individual functioning in a group, each participant's relation with a patient or the role of the leader of a social group);
2. The quality of patient — doctor relation, the causes of any difficulties in patient — doctor relation such as excessive sense of responsibility, differences in patient's and doctor's hierarchy of values, attempts to create a partnership, doctor's inability to accept failure in therapy, empathy, projecting one's problems or perceived failure.
3. Awareness of the changes taking place (i.e. changing knowledge, patient's, patient's family's or other worker's changing attitude to cooperation),
4. The emotions that arise.

Additionally, there were three open questions which were related to 1. most important emotions evoked during workshops; 2. the value of group work for each student and 3. negative consequences of this work. Students participating in the study were informed that the results of the study will be published and gave their full consent for the publication.

Results

All students participating in the study completed the questionnaires.

1. Almost 90% of the subjects reported that they gained knowledge on social interactions, particularly with reference to group functioning and their relations with patients (**Figure 1**).
2. Participants of the study reported major difficulties related to a sense of excess responsibility and corresponding problems in accepting therapeutic failure (**Figure 2**). Interestingly, their answers to subjects answered negatively to the other items of the questionnaire were negative. One may conclude here that respondents either found it easy to deal with or could not admit their difficulties related to projection of their own problems, striving for dominance and partnership.
3. Almost all students participating in the study agreed that during the workshop they gained insight into psychological aspects of patient — doctor relationship and their attitude to patient has changed. Students especially emphasized that presently they were able to understand their behavior much better (86,57%).

Additionally students answered three open questions. Their answers to these questions are presented below.

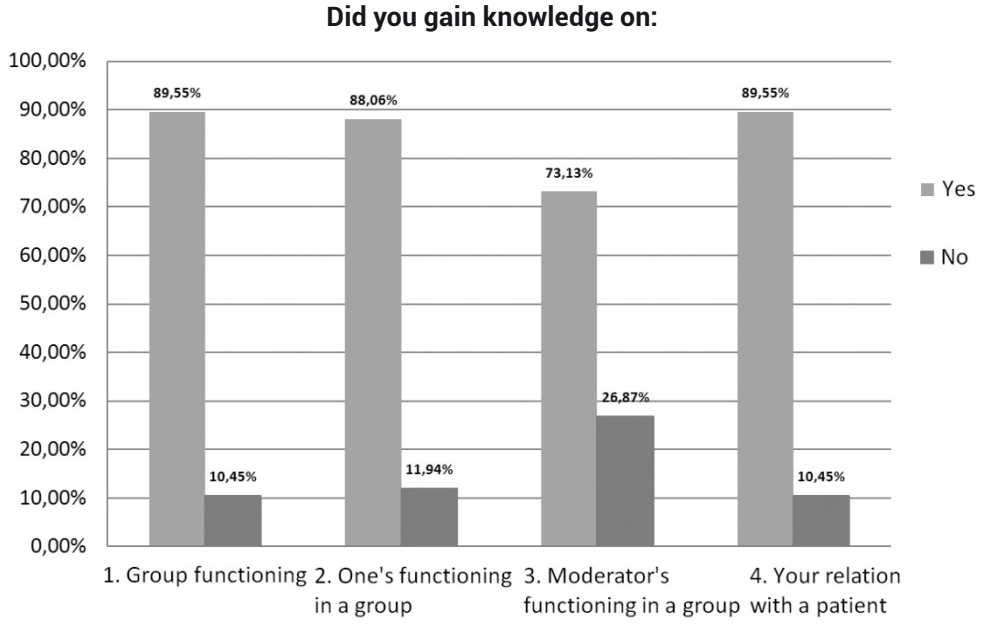

Figure 1. Student's knowledge about the group process

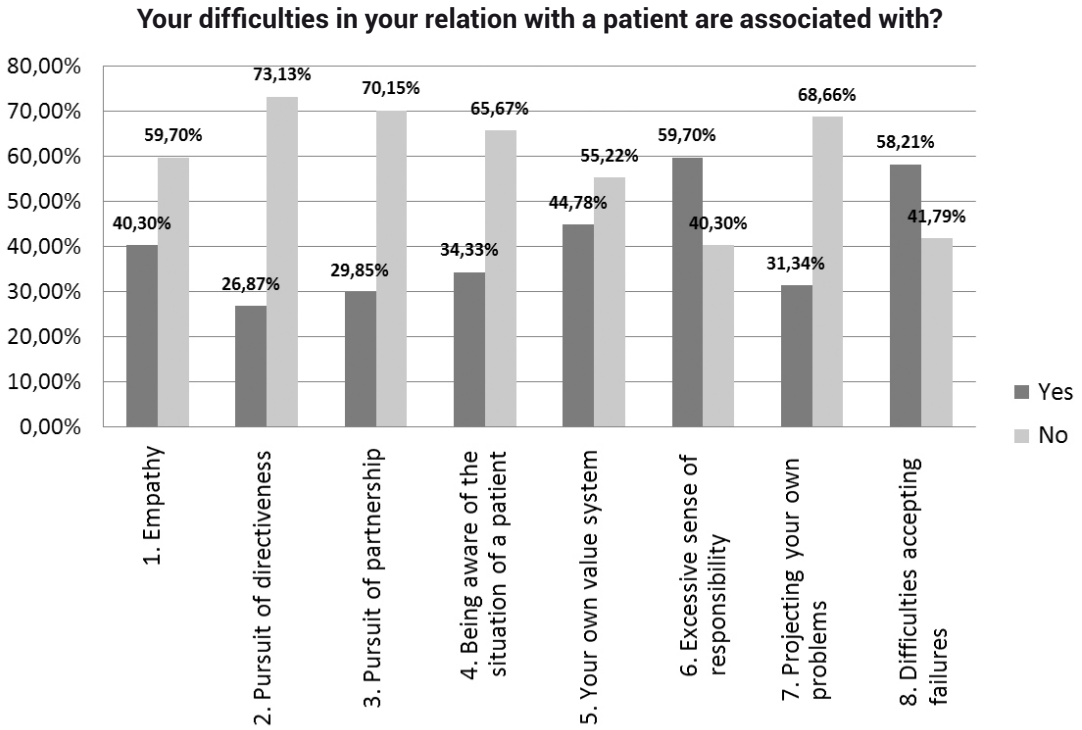

Figure 2. Quality of medical student – patient relationship and their insight into difficulties in this relationship

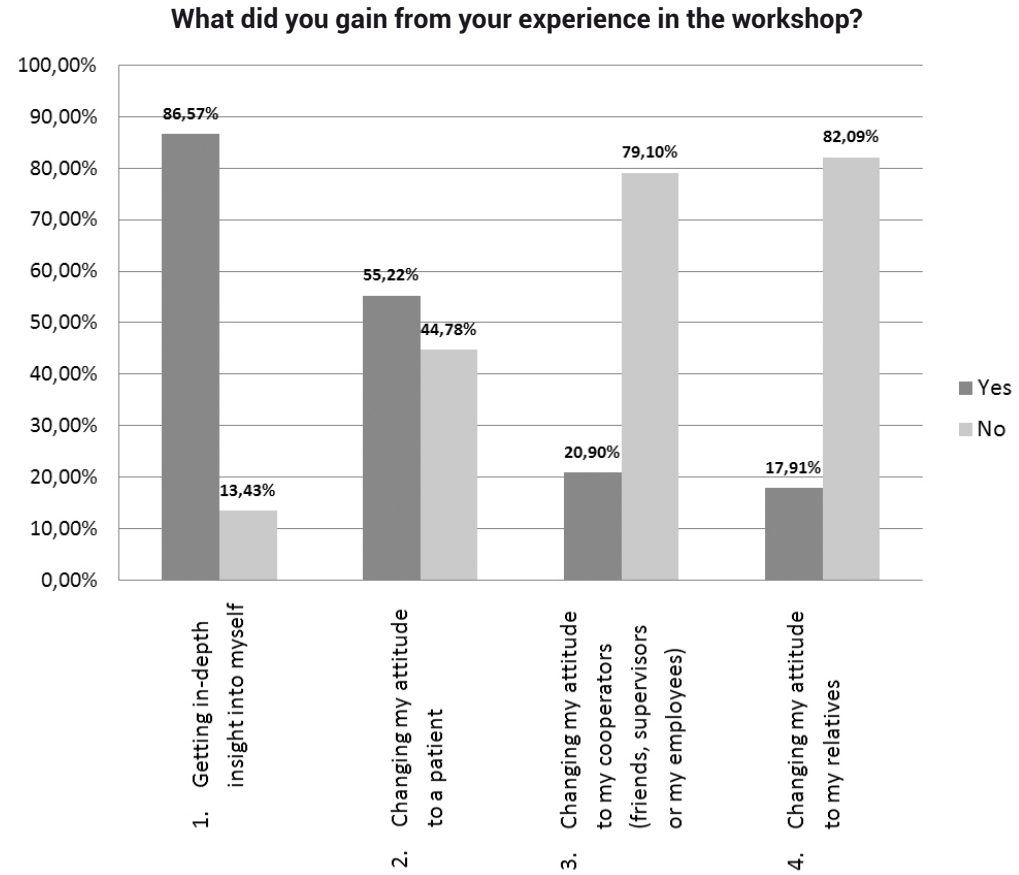

Figure 3. Student's awareness into changes following Balint's group workshops

What was your greatest emotional experience during the workshop?

- Analysis of a seemingly easy case report, which had a lot of aspects. Setting clear borders in the doctor — patient and doctor — nurse relation.
- Listening to someone else's opinions on the case report i presented.
- Discovering that sometimes it is enough to think a little to help the patient.
- Uneasiness due to lack of one's own case for presentation, i know that the relation with a patient may be difficult but i don't remember anything, i think that my relation with patients is "shallow".
- Awareness that patients do not come to us to irritate us and it is not their goal to trigger doctor's negative emotions.
- A sense of powerlessness (there are so many patients whom we cannot help).
- Adopting patient's point of view, finding a way to understand the patient's situation better ("getting into patient's shoes").
- Finding new solutions to the same problem.
- Finding one's heart to talk in public and to present one's point of view.
- An opportunity to pay attention to a patient — doctor relation if the patient is a person with a disability.
- Empathising with the patient, seeing the patient from a different perspective.
- Helplessness in the face of discussed problems.
- An opportunity to describe my patient and my changing perception of my patient.
- Participating in a discussion.
- A conversation on "getting to know" the patient better.
- Being able to understand that each patient's or doctor's behavior is adequate for the situation and explicable
- An attempt to empathize with a doctor whose patient died.
- Feeling unable to influence emotions of parents of chronically ill children.
- A conversation on pathological situation in a patient's family.
- Being able to notice one's emotional problems.
- Being able to understand the importance of talking to a patient and getting to know him/her better.

What was important for you in group work?

- The fact that everyone could talk openly about difficult and painful issues.
- Everyone was committed, no one avoided difficult themes, everybody showed respect to other points of view.
- Mutual kindness and openness.
- The fact that each group member could share their opinions, thoughts or observations.
- Cooperation, an opportunity to share feelings and emotions, being able to recognize how others perceive the relation with a patient.
- Changing attitude to a patient and patient — doctor relation; the emotions changed completely after the case report had been discussed in a group and after each stage of work at a balint's group meeting.
- Being able to see other's point of view and willingness to realize my own point of view.
- Presenting one's opinion on patient's emotional and psychological status.
- Having an opportunity to obtain answers to bothering questions.
- Confronting my point of view with other opinions.
- Group cooperation to solve patient's.
- Group activity, diversity of ideas.
- Openness, straightforwardness, looking for patient's best interest.
- An opportunity to learn someone else's attitude to patients.
- Listening to other opinions without making any evaluations.
- A right to have one's own opinion and to make one's individual interpretation.
- Sharing one's emotions with others.
- Learning about the mechanism of transference and countertransference.
- The fact that everything was secret as a rule, we could tell about our experiences, share opinions and open ourselves to others.
- We could broaden our minds by learning other opinions.
- Being able to understand that our interpretation of patient's behavior does not always reflect what the patient feels.

What were the negative consequences of participation in a workshop you were able to observe?

- Exhaustion, too long meetings.

- Inclination to complain or to gossip.
- I was irritated by the fact that not everyone understood the idea of meetings.
- I related the situation to myselftoofrequently.
- I had difficulties formulating the problem.
- Nervousness.
- It was pretty stressful to analyze one's.
- Letting off steam.
- I probably confessed to much in the heat of the moment, people who are strangers do not have to know my feelings and experiences to such a high extent. I could have said more than others and then i felt a little silly.
- Being withdrawn and uptight, feeling embarrassed.
- High emotionality.

Discussion

There are relatively few studies on Balint groups in Poland apart from the results of Jugowar and Skommer's presented at International Balint congress in Stockholm in 2005, which showed that the workshops are advantageous for students and their future patients [6]. Interestingly, students need time to ascertain the value of workshops because the longer the workshops the higher their level of satisfaction [7]. The effectiveness of Balint's workshops was confirmed by several studies globally [8, 9], e.g. in groups of Finnish medical students [10], in Argentina [11] or in Germany, where all medical doctors participate in Balint's workshops [12, 13]. Workshops seem necessary because sheer knowledge on psychosomatic illnesses does not reduce powerlessness in the face of patient's symptoms and complaints. Medicine and psychotherapy are a craft which can be mastered while practicing or observing mentors. Balint developed a system of training for doctors, which was based on group work. Workshop participants are doctors of diverse specializations therefore group members differ in their ways of patient management. Additionally, exchange of experience may point to new aspects of the discussed cases. Group participants are encouraged to discuss cases where aspects of patient — doctor relation do not go as expected. The main value of this method relates to the fact that group participants have to understand patient's and doctor's feelings and thoughts and learn alternative behaviors. The atmosphere is usually undisturbed, friendly and characterized by solidarity.

Consequently, the following skills which are useful in building adequate patient- doctor relations can be developed:

- better understanding of patient's situation or his social and family problems,
- the knowledge of patient's behavioral patterns,
- the ability to recognize patient's resistance,
- conflict situations and related emotions,
- awareness of the importance of symptoms presented by patients and his /her expectations about doctor
- the ability to recognize difficulties in the patient — doctor relation.

The results of the study and the observed changes may be considered remarkable because they followed participation in a 10-hour workshop training. Specific skills which improve patient — doctor relationship may play a significant role not only in building and maintaining contact with the patient but can also assure correct diagnosis. This can be particularly important for psychosomatic patients, who make 20–25% of all patients who seek advice of a general practitioner thus generating high costs of treatment [14]. Rapid benefits for students should be taken into consideration in the medical student education because this method is a common or even obligatory part of curriculum in many countries.

Physicians need to understand mechanisms leading to health disorders in order to be able to reconize the relation between patient's emotions and their somatic functioning. The advantages of participating In Balint's workshops are mainly related to increasing ability to solve characteristic problems in the patient — doctor relation, which arise in three areas i.e. difficulties related to patient's current health status. Patients with illnesses that are difficult to diagnose or with a bad prognosis are more likely to poorly communicate with their doctors. As a result, doctor's appointments may become shorter or physicians try to avoid seeing these patients. Paradoxically, doctors try to explain the situation by patient's good or patient's right to intimacy. In fact, these patients are perceived as difficult because they are likely to ask difficult questions and may trigger difficult emotions. The same rule applies to

pediatric patients i.e. seriously ill patients less cared for by medical personnel.

The second type of difficulties lies with the patient. We have to bear in mind that patients remain human beings who can be anxious, depressive, demanding, competitive. They may read also too much about their illness, are non — compliant or refuse to participate in treatment. The third type of difficulties lies with the doctor, who may have a perfectionist attitude to treatment or diagnosis, may react with anxiety or remain non-emphatic and directive to patients. These problems are analyzed during Balint's workshops. The benefits for the doctor, which include insight into one's feelings and their analysis, increasing empathy and ability to recognize and control transference and counter-transference, lead to more comfortable work and increasing job satisfaction. These new competences should be treated as instrumental as they increase the quality of service to the patient thus smoothing over any difficulties.

Implications for practice

Balint group participants learn to look at the world with their patient's eyes. As a result, students are more inclined to more elastically change their point of view and are able to see patient's situation and their relation in a wider context. Students are taught about mechanisms and processes of contact so they are able to understand by acknowledging their own emotions and by shaping new behaviors based on theoretical principles of group dynamics. To add, students are not given ready — made answers on how to behave with a particular patient but they learn what other group participants and the group leader think about the situation. Consequently, the classes may sometimes evoke extreme opinions

Acknowledgements

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
There are no sources of funding to declare.

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Acceptance for editing: 2019-07-23
Acceptance for publication: 2019-09-15

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ORIGINAL PAPER

DOI: https://doi.org/10.20883/jms.366

Original educational computer program "Trauma" and its application in education of paramedic students – preliminary results

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ABSTRACT

Education through simulation is becoming increasingly popular in medical academic environment. This is the best teaching method enabling the creation of real situations in risk-free conditions. Decision-making games can be successfully used in the educational process of future medical staff. The aim of the work was to create a didactic computer program "Trauma", analyze its impact on students' knowledge on the direction of medical rescue and evaluate the attractiveness of classes conducted with the use of this method. The results show that the use of the "Trauma" program in didactics has allowed for the improvement of the level of knowledge and skills of students taking part in the study in the field of trauma patients' treatment. In the assessment of students, the classes during which the program was used were interesting. The vast majority of respondents would like to participate in such classes again.

Keywords: trauma patient, didactics, medical rescue.

Introduction

A significant development of industry, mechanization of work and means of communication affect the increase of number of trauma among all societies in the world [1]. Injuries which result from trauma often lead to permanent disability or death of the victim. This represents an enormous burden both for individuals and the society at large. In Poland, trauma is the third leading cause of death and in case of children and adolescents (5–19 years) together with poisoning they account for more than a half of all deaths [2, 3]. The most common causes of trauma are traffic accidents, falls, aggression and work-related accidents [4, 5].

Trauma patient constitutes a great challenge for the staff that brings him/her aid, especially for paramedics who often need to implement life-saving treatments at the scene of the accident. The limited equipment they have, potential risks in the area of their operation and

limited human resources are just some of the obstacles. The sole assessment of the condition of trauma patients can also be difficult. That is why a systematized action plan becomes so important [6]. Providing help in a stressful situation requires not only good theoretical preparation but also significant practice. Paramedic students the opportunity for practical application of the theory during medical simulation. Its origins date back to 1960, when Laerdal Company launched manikins used for training of CPR — "Resusci Annne". Currently, there are many advanced phantom models available on the market that allow people to learn and practice almost every medical procedure used in emergency medicine. Universities which educate medical personnel create simulation centers where students can gain knowledge without endangering health and life of patients. Unfortunately, these are costly investments. It is also necessary to arrange the place, adapt the premises and employ additional teaching staff. There is also a limitation on the number of participants who can take part in such classes. Computer simulators are becoming a solution to these problems. They give people an opportunity to learn both basic procedures and complicated treatments. One of the more and more willingly used tools are decision-making games [7, 8].

The original educational program "Trauma" presented in this study is a training method in the field of assessment of a trauma patient, his qualification for a trauma center and emergency medical services performed. Individual tasks of the program are presented in the following bullet points:

- Patient evaluation using the Glasgow scale.
  Information necessary to perform this task (motor response, verbal contact, eye opening) was included in a patient's card. The program required assigning the injured person an appropriate number of points on the GCS scale and, on their basis, identifying possible disturbances of consciousness.
- Trauma recognition
  The educational program "Trauma" enables simulation of trauma which could be sustained by a trauma patient. One of the tasks was a preliminary diagnosis on the basis of information from patient's image and the value of additional parameters, including breath count, heart rate, blood pressure, peripheral and carotid artery pulse, and capillary refill time. Each of the simulated patients had three injuries which the program assigned in a random order.
- Emergency medical services
  Each trauma from the database of the "Trauma" program has some specified procedures that should be performed at the scene of the incident and during the transport of a patient. Based on this, emergency medical services were analyzed. The correctness of the implemented emergency medical services was assessed on the basis of a five-point scale
- Qualification to a trauma center
  On the basis of the overall analysis of data included in a patient's card, the program required determination of a decision regarding qualification or non-qualification of a patient to a trauma center.

Assumptions and aims of the study

Own experience has led to the conclusion that training in the field of approach to a trauma patient is insufficient. Although topics concerning this matter are thoroughly discussed theoretically, they are not fully absorbed by students due to the limited possibilities of practical exercises.

Keeping in mind the improvement of quality of education of paramedic students in the field of trauma patient treatment, an attempt was made to create an educational computer program that would allow for practical training, using the available means, of a large number of people.

The aim of the study was:

- The development of an educational program called "Trauma" which enables the improvement of knowledge and skills of paramedic students in the field of treatment of trauma patients.
- An assessment of the impact of the "Trauma" program on the knowledge of students in the field of approach to trauma patients, with particular emphasis on the assessment of the state of consciousness in the Glasgow scale, initial recognition of injuries and qualification of patients for treatment in a trauma center.

› Assessment of satisfaction of students participating in the study from classes conducted using the educational program "Trauma".

Material and Methods

The study was conducted in the period from 1 January 2016 to 25 April 2016 in a group of 75 students of paramedics students of Silesian Medical University in Katowice. There were 45 (60%) women and 30 (40%) men in the study group. The respondents were students of the first and second year of bachelor, full-time studies.

Educational classes with the use of the "Trauma" program were conducted in three recurring stages. Each of them consisted of a two-hour course, during which students were acquainted with the legal basis of performing the profession of a paramedic and they actively participated in practical exercises realized with the help of the educational computer program "Trauma". The end of the exercises was a lecture summarizing the achieved results and a short discussion about the conducted simulation.

The purpose of the exercises with the use of the "Trauma" program was to introduce students to the issue of multiple and multi-organ injuries. The task of people who participated in the study was to assess the state of consciousness of a patient according to the Glasgow scale, an initial diagnosis of injuries the patient experienced and the qualification or disqualification for treatment at a trauma center. The exercise cycle was based on scenarios, where a total of 225 victims were simulated on the assumption that each of them suffered from three injuries. All necessary information about the patient's condition was included on the patient's card automatically printed after the program drew particular injuries. The exercise cycle was completed by a student satisfaction survey from conducted classes during which the educational program "Trauma" was used.

Statistical analysis

Quantitative variables were presented in the form of frequency and percentage of individual categories. Statistical significances of differences among groups were verified by χ^2 test. Cochran-Armitage's test was used for trend assessment in multi-area tables.

Analyzes were carried out in a statistical package Statistica (version 12) and R (version 3.2.3).

$\alpha = 0{,}05$ is the level of statistical significance that was assumed.

Results

Assessment of patients in the Glasgow scale

The first element analyzed during the simulations with the use of the educational program "Trauma" was patient's assessment using the Glasgow scale. The information needed to complete this exercise (motion response, verbal contact, eye opening) was included in a patient's card. The task of the students was to assign the injured person an appropriate number of points in the GCS scale and to identify on this basis any possible disturbance of consciousness.

Among the 225 injured people simulated by the program 103 (45.8%) presented mild disturbances of consciousness, ranging from 13 to 15 points in the Glasgow scale. Moderate disturbances of consciousness (12–9 GCS points) were present in 84 (37.33%) patients, unconsciousness (8–6 GCS points) in 31 (13.8%), decortication (5 GCS points) in 5 (2,2%), and decerebration in two (0.9%) patients. None of the simulated victims showed brain death (3 GCS points).

During the first exercise cycle, the correct score in the Glasgow scale for simulated trauma patients was given by 42 (56%) students. Even if students correctly calculated the sum of GCS points they had a problem with determining the degree of consciousness disturbance. Only 31 (41.3%) of the respondents reported the correct classification. In the second exercise cycle, the correct score in Glasgow scale was given by 49 (65.3%) of the students. This shows an increase in correct responses by 9.3% in relation to the first exercise cycle. What is more, in this case 55 (73.3%) of the participants of the study correctly classified consciousness disorders. Therefore, an increase was noticed in the correct classification of consciousness disorders by 32% in relation to the first study. Students who took part in the study in the last, third cycle of exercises in a vast majority presented knowledge of the score of patient's consciousness in Glasgow scale and the classification of consciousness disorders. The correct sum of GCS points

was given by 65 (86.7%) of the respondents, while the consciousness disorders were correctly classified by 68 (90.7%) students. This shows that in the third exercise cycle with the use of the educational "Trauma" program, the number of correct responses in relation to the second exercise cycle increased by 21.3% in case of the assessment of points in the Glasgow scale and by 17.3% in the classification of consciousness disorders (**Figure 1**).

In no exercise cycle, statistically significant differences were observed in the distribution of the correct answers between the sum of points of the scale GCS and classification of the disturbances of consciousness. During the research we could observe statistically significant growth of the correct students' answers in each subsequent exercise cycle.

Trauma diagnosis

For the purpose of the analysis of correctness of initial diagnosis made by the students, each trauma was counted separately, giving 225 different

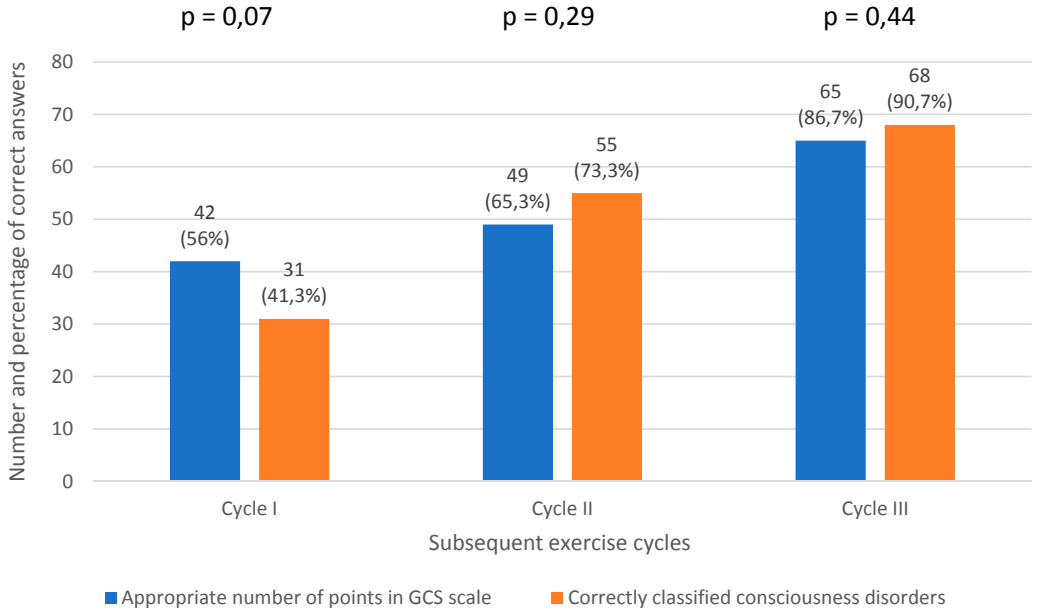

Figure 1. Number and percentage of correct responses concerning consciousness disturbances in the Glasgow scale in subsequent cycles

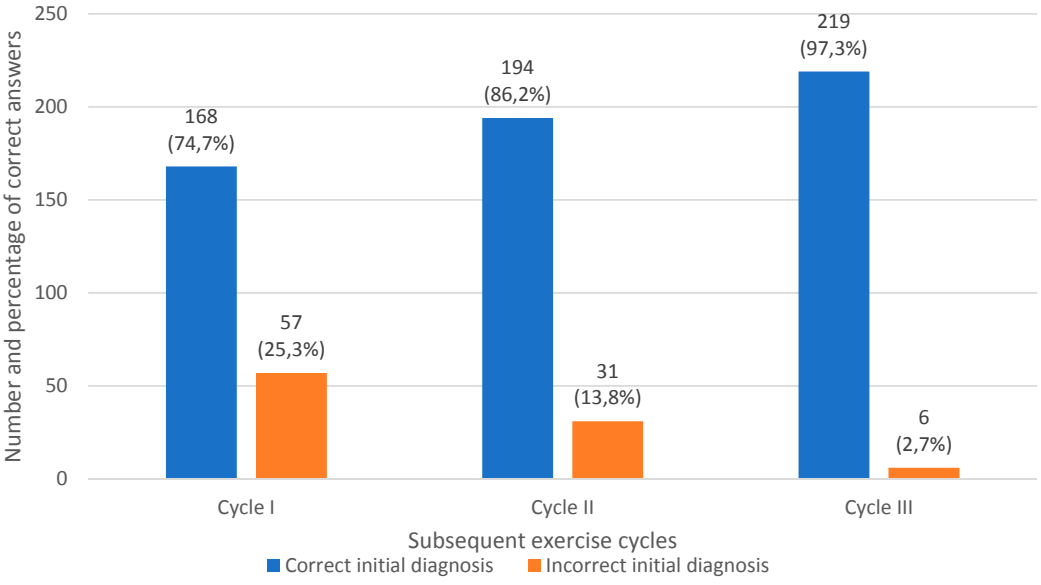

Figure 2. Correctness of the initial diagnosis made by students taking part in the study in each cycle of exercises ($p < 0,01$; Cochran-Armitage's test)

injuries in each exercise cycle and 675 simulated injuries during the three exercise cycles. During the first exercise cycle, a correct initial diagnosis was made in case of 168 (74.7%) injuries. The second exercise cycle showed an increase in the number of correctly diagnosed injuries. In this case, the correct diagnosis was made 194 (86.2%) times. The upward trend was maintained in the third exercise cycle, during which 219 of 225 injuries (97.3%) were correctly diagnosed. The second cycle of exercise with the use of the "Trauma" program showed an increase in the number of correct initial diagnoses of injuries in relation to cycle 1 by 11.6%. In the third cycle in relation to the second cycle, an increase was noted in the number of correct diagnoses posed by the students by 11.1%. A Cochran-Armitage's trend test confirms the above observations (**Figure 2**).

Qualification for treatment in a trauma center

According to the statistics of the "Trauma" program among 225 simulated patients 118 (52.4%) should be qualified for treatment in a trauma center. The first cycle of exercises showed significant errors made by the students in qualification of a trauma patient to a TC. Among 75 victims simulated by the program in the first cycle of training, only 12 (16%) were correctly qualified or disqualified for treatment at a trauma center. This result improved during the second exercise cycle. Correct classification or correct disqualification, in this case selected 32 (42.7%) of the students. The upward trend of correct responses was maintained in the third cycle of exercises during which as many as 68 (90.7%) students correctly qualified or correctly disqualified simulated patients for treatment in a TC. A Cochran-Armitage's trend test confirms the above observations (**Figure 3**).

Satisfaction of students from the exercises

The overwhelming majority of participants of the study expressed a positive opinion about the "Trauma" program and would like the classes to be run in this form more frequently. Among the students taking part in the study, 92% gave the "Trauma" educational program the highest possible rating — 5. The respondents were also asked about the potential of the program in education and their willingness to participate in classes conducted with the use of the program. The overwhelming majority of respondents (96%) think that classes conducted with the use of the educational program "Trauma" were interesting and they were willing to participate in them again. Students were also interested in making the program available for individual use for simulation exercises outside of educational classes (**Figure 4**).

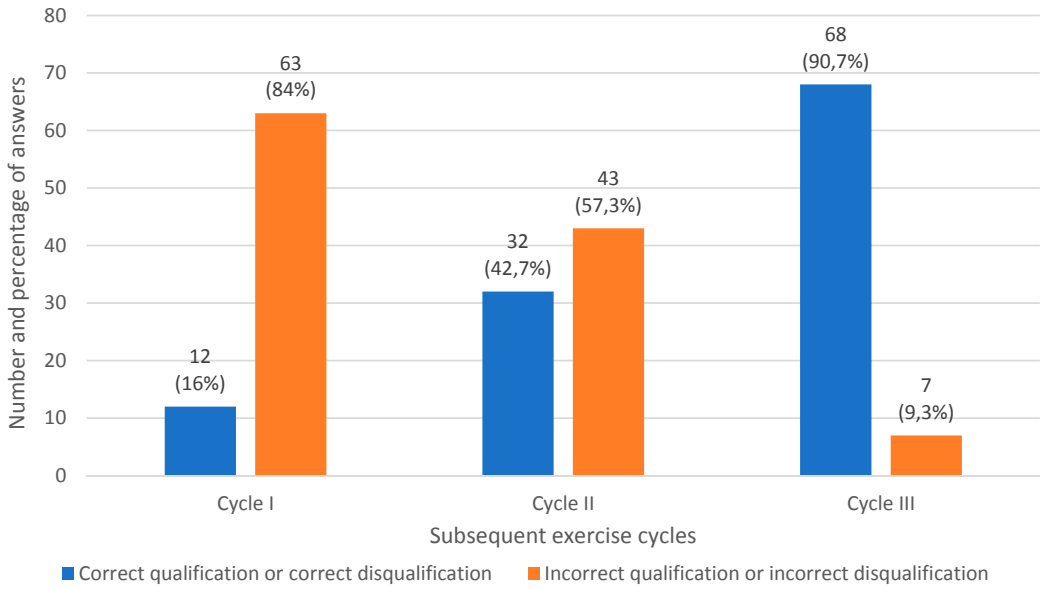

Figure 3. Correctness of qualification or disqualification of simulated patients for treatment in a trauma center in following exercise cycles ($p < 0,01$; Cochran-Armitage's test)

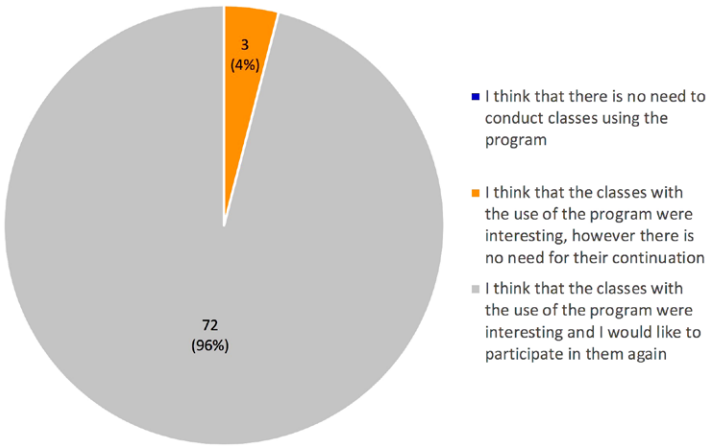

Figure 4. Opinion of students participating in the study about conducting classes with the use of the "Trauma" program

Description of results and discussion

During the three exercise cycles with the use of the educational program "Trauma", there was a clear increase in the number of correct responses given by students. During the training of assessment of consciousness disorders in the Glasgow scale in trauma patients there was an increase by 30.7% of the correctly allocated GCS score in the third exercise cycle in comparison with the first and an increase by 49.3% in the correct classification of consciousness disorders. Training of preliminary diagnosis of trauma basing on symptoms and assessment of physiological parameters showed an increase in correctly diagnosed injuries by the students in the third exercise cycle in comparison to the first by 22.7%. The most effective were exercises in qualification of a patient to a trauma center. In this case, the number of correct qualifications or disqualifications increased by 74.7% in the third exercise cycle in comparison to the first cycle. Students actively and with great engagement participated in classes with the use of the "Trauma" program. Simulation exercises were considered interesting and the program itself was mainly given the highest possible rating. Among the study group, 96% of students would be willing to participate in next classes with the use of the program.

The efficiency of teaching depends to a large extent on how knowledge is transferred. Medical simulation based on modern technology is considered by many authors to be the best tool for training medical staff whose primary purpose is to shape good patterns of behavior. Simulation training is designed to help the future medical staff to adapt to difficult working conditions that differ greatly from the lecture room.

The textbook, published in 1664, describes the use of games during the training of the Prussian army. War games were developed and widely used as training tools for the army until the mid-twentieth century [9]. Significant development of decision-making games was influenced by their use in the training of management personnel of production companies in the Soviet Union and the USA. Currently, these games are widely used in managerial training, where leading a team and making difficult decisions are crucial. Similar situations also apply to the work of a paramedic and that is why decision-making games can be successfully used in training of future paramedics. Numerous studies confirm the effectiveness of using this method in training of medical personnel [10, 11, 12].

In the twentieth century computer programs based on the principles of decision-making games started to be introduced into medical education, but still the dominant form of knowledge transfer was the lecture [10].

Education through simulation is becoming increasingly popular in the academic community. In addition to valuable knowledge gained from books or the latest scientific reports, computer simulations are becoming an increasingly effective form of education. Simulation teaches to transfer the acquired theoretical knowledge onto practical action. By means of multiple repetitions of a given simulated situation, it gives the opportunity to cre-

ate psychomotor skills performed in later practical actions in an automated manner [15].

Huang et al. noted that a valuable teaching tool is especially the test environment, which allows learners to acquire the skills of making many complex decisions in a short time and thereby to work in stress.

Frączek and Cebula in their publication also pointed out the need for medical simulations, which are designed to improve work in crisis situations. In their study they refer to the report of the Medical Institute of the United States of North America from 2000. According to it, in the United States 44,000 to 96,000 people die annually due to medical malpractice, mostly resulting from the staff's difficulty in transferring knowledge into action in very stressful conditions. This publication has influenced the growth of importance of training of medical staff with the use of appropriately adapted simulation stations [13].

The effectiveness of education conducted with the use of decision-making games was also appreciated by Specht and Sandlin. In their study they compared the effectiveness of traditional training led in the form of a lecture with training based on simulation management game. Participants of the study were divided into two groups — one trained through a decision-making game, the other group consisted of people attending lectures. Evaluation of the effectiveness of both trainings was made through tests checking the acquired knowledge, which the participants solved after a longer period of time. This study unequivocally confirmed the superiority of training with the use of a managerial game [14].

Sowizdraniuk et al. conducted a study on a group of 46 second-year paramedic students aimed at assessing the effectiveness of teaching. Students taking part in the study were divided into three groups and attended four-hour classes concerning advanced cardiopulmonary resuscitation and procedures in case of bradycardia and tachycardia. Classes of each group were conducted by different teaching methods: lecture, case study and simulation game. The effect of the conducted classes was measured by a standardized knowledge test concerning the discussed topics held after one month. The highest efficiency of teaching was achieved in the group of students participating in the simulation game. The respondents in this group answered fastest to the questions they were asked, which, according to the authors, could indicate easier decision making. In a subjective assessment of classes most points awarded by the students were obtained by the ones conducted with the use of simulation games. The results obtained by Sowizdaniuk et al. show highly effective teaching with the use of simulation games and the attractiveness of classes conducted in this way, which may cause greater motivation of students to acquire knowledge [10].

Traditional methods of education, especially in case of students of medical universities, seem to be insufficient. More and more attention is being paid to ways to increase the effectiveness of learning. One of them is medical simulation. Young people growing up in a multimedia environment are eager to use electronic methods to improve their knowledge. Trainings conducted with the use of medical simulation based on decision--making games allow not only for development of appropriate habits used later in real life. They also allow people to identify committed mistakes, which in turn may lead to their minimization during real activities. In addition, it is a form of education that is pleasant for participants and that is why they are more likely to engage in classes.

Decision-making games can also be effectively used in exercises for a well-trained staff in order to prevent forgetting skills due to lack of their repetition. This is particularly important in case of medical staff on whose knowledge and skills depends the patient's life. The increasingly popular method of training medical personnel through simulation allows to shorten the learning process while achieving greater efficiency at the same time. It also protects against damage done to real patients while learning, for example, on an internship. The results of many studies on the use of educational games confirm the effectiveness of this educational method, which is appreciated by the participants of the training sessions in which it is used.

Educational program "Trauma" allows for learning how to recognize trauma that patients have experienced, how to properly evaluate their physiological parameters and to qualify them for treatment in a trauma center. It is a cheap method which is also easy-to-use during educational classes regardless of where they are carried out.

It does not require specialized equipment or costly phantoms. In a short time it allows you to train a large group of participants. Thanks to the ability of expansion and modification of the injury database, there is an unlimited number of simulated patients.

Conclusions

1. The creation and implementation of the educational "Trauma" program has made it possible to increase the level of knowledge and skills of paramedic students in the area of trauma patient treatment.
2. Exercises conducted with the use of the educational program "Trauma" showed an improvement of abilities of the examined group of paramedic students in evaluating trauma patients, diagnosing injuries and qualifying patients for treatment in a trauma center.
3. In the assessment of students participating in the study, the classes conducted using the educational program "Trauma" were interesting. The vast majority of respondents would like to participate in such classes again.

Acknowledgements

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
There are no sources of funding to declare.

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Acceptance for editing: 2019-07-23
Acceptance for publication: 2019-09-15

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ORIGINAL PAPER

DOI: https://doi.org/10.20883/jms.311

Mass spectrometry analysis of redox forms of High-Mobility Group Box-1 Protein in cerebrospinal fluid: initial experience

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ABSTRACT

Introduction. High-mobility group box 1 (HMGB1) is an alarmin with proinflammatory potential determined by redox status of the cysteines at position 23 and 45. It may also play a role as a biomarker in biological fluids. The aim of this study was the identification of different HMGB1 redox forms in cerebrospinal fluid (CSF) obtained from subarachnoid hemorrhage patients.

Material and Methods. 6 CSF samples were collected from aneurysmal subarachnoid haemorrhage patients. Commercially available HMGB1 isoforms served as a positive control. Immunoprecipitation and electrophoretic isolation of HMGB1 protein were performed, then both CSF and control were analyzed using mass spectrometry technique. To distinguish between fully reduced (thiol group at C23 and C45) and disulfide (disulfide bond connecting C23 and C45) HMGB1 forms, top-down sequencing of the spectra was performed.

Results. Top-down sequencing analysis allowed to distinguish between HMGB1 isoforms only in commercially available standard without preceding immunoprecipitation and electrophoresis. MALDI spectra differ i.e. on the fully reduced HMGB1 spectrum fragmentation occurs before and beyond C22, which is not present on the disulfide HMGB1 spectrum. Analysis of HMGB1 isolated from CSF obtained from subarachnoid hemorrhage patients gave no results.

Conclusions. Top-down sequencing enables to distinguish between redox forms of HMGB1. Electrophoresis and tryptic digestion cannot precede mass spectrometry analysis of redox forms of HMGB1 due to the reduction of disulfide bonds during these processes. Preferred method of isolation of HMGB1 for direct analysis using top-down sequencing mustn't include protein digestion or degradation.

Keywords: HMGB1 protein; cerebrospinal fluid; MALDI; mass spectrometry; top-down sequencing; redox.

Introduction

High-mobility group box 1 (HMGB1) protein (also known as amphoterin) was discovered in 1973 and first described as a non-histone chromosomal protein and a regulator of gene transcription [1, 2]. Further studies revealed involvement of HMGB1 in pathogenesis of the inflammatory and autoimmune diseases. In 1991 Wang et al. identified HMGB1 as a late mediator of endotoxin lethal-

ity in the mice [3]. What is more, the same article described HMGB1 therapeutic potential as administration of a HMGB1 blocker increased survival rates. Currently, we are aware of HMGB1 involvement in such common diseases as myocardial infarction [4], cerebral ischaemia [5], arthritis [6] and trauma [7]. Both wide expression and diversity of biological actions has led to increase in popularity of HMGB1. Over the last decade number of publications mentioning HMGB1 has increased 3 times (2007: 182 articles; 2017: 633 articles; according to www.ncbi.nlm.nih.gov/pubmed). Our own interest focuses on the role of HMGB1 in biological fluids as a biomarker. To extracellular space HMGB1 can be released from the cell interior by both passive (e.g. during necrosis) and active (i.e. secretor vesicles) routes [8]. Outside of a cell HMGB1 binds to the pattern recognition receptors (e.g. Toll-like family) and triggers stereotypical inflammatory reaction associated with increase of interleukin 6 and tumor necrosis factor α in the neighbour cells [9]. Based on our own experience, HMGB1 can be used as a prognostic biomarker in the subarachnoid haemorrhage (SAH) patients [10]. Nevertheless, in our study we have encountered patients lacking correlation between high HMGB1 level and unfavourable outcome. This might be explained by the differences in redox status of assayed HMGB1. Currently, we are aware that redox status of the three cysteine residues (position 23, 45, 106) determines the extracellular role of HMGB1 [11]. The fully reduced (all-thiol) HMGB1 form induces chemotaxis, the fully oxidized form (sulfonyl HMGB1) has no known function, while form of the intermediate redox status (disulfide HMGB1) induces cytokine production [12]. In the clinical scenario the balance between reactive oxygen species and natural antioxidative enzymes (glutathione peroxidase, catalase, and superoxide dismutase) determines proinflammatory potential of the HMGB1 by influencing its redox status [13, 14]. Unfortunately, the most popular method used to assay HMGB1 level in the biological fluids (enzyme-linked immunosorbent assay) is not capable of distinguishing between forms of HMGB1. Mass spectrometry on the other hand, may be used for HMGB1 redox forms identification [11, 14]. The aim of this study is isolation from the cerebrospinal fluid (CSF) and identification of different isoforms of HMGB1 protein using proteomic tools.

Material and Methods

Material

Patients suffering from SAH due to intracranial aneurysm rupture were included in the study. CSF samples were collected on day 0–3, 5 and 10–12 from external ventricular drainage implanted as a treatment for coexisting acute hydrocephalus. After centrifugation samples were stored at -70 Celsius degrees. Patients' treatment outcome was assessed at 6 months. For the analysis we have selected two model patients (no severe comorbidities, typical demographic for SAH) of various treatment outcome (favorable and unfavorable). CSF samples collected at all time points (day 0–3, 5 and 10–12 after aneurysm rupture) were assayed. Commercially available disulfide HMGB1, lipopolysaccharide (LPS) free (HMGBiotech, Milano, Italy) and fully reduced HMGB1, LPS free (HMGBiotech, Milano, Italy) were acquired as standard for comparison with biological samples as well as true positive control.

Matrix assisted laser desorption/ionization (MALDI) top-down sequencing

Disulfide HMGB1, LPS free and fully reduced HMGB1, LPS free were dissolved in 0.1% trifluoroacetic acid (TFA) in water. Matrix — 1,5–Diaminonaphtalene (1,5-DAN) (Bruker Daltonics, Bremen, Germany) substance was saturated in TA50 solvent (mixture of acetonitrile: 0.1% TFA in water, 50:50). 2 µl of matrix solution was mixed with 1 µl of sample solution (disulfide HMGB1 and fully reduced HMGB1). 0.5 µl of the prepared mixtures were spotted onto the Ground Steel MALDI Target Plate and left to dry at room temperature. Analysis was conducted using MALDI-Time-of-Flight (TOF) mass spectrometer (UltrafleXtreme, Bruker Daltonics, Bremen, Germany) and ion-source decay (ISD) fragmentation technique in the mass range of 1–8 kDa. The mass spectra were externally calibrated using Bovine Serum Albumin (BSA) standard and average mass deviation was below 100 ppm. Acquisition and spectra processing were performed by FlexControl 3.4 software (Bruker Daltonics, Bremen, Germany). Evaluation of the spectra was performed in FlexAnalysis 3.4 software (Bruker Daltonics, Bremen, Germany).

Immunoprecipitation and analysis of HMGB1 isolated from the cerebrospinal fluid

Full workflow is presented on **Figure 1**. Each assay step is described in detail below.

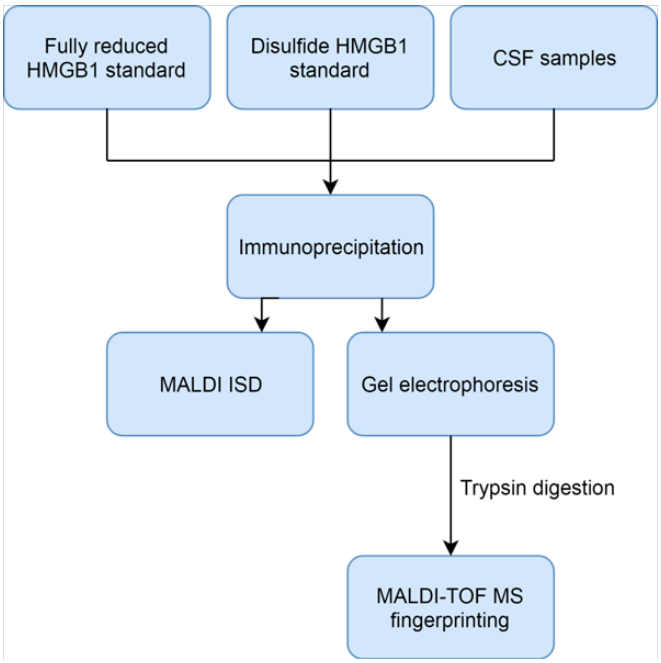

Figure 1. Workflow of immunoprecipitation and analysis of fully reduced HMGB1, disulfide HMGB1 and CSF samples

Immunoprecipitation

Immunoprecipitation procedure was conducted according to protocol provided by Sigma-Aldrich — method A [15]. Briefly, Protein G Sepharose (Sigma Aldrich, St. Louis, Missouri, United States) was washed twice and resuspend with washing buffer (PBS pH 7.4). Agarose conjugate was divided into 90 µl aliquots. Next, 10 µl of primary antibody Anti-HMGB1 antibody produced in rabbit (Sigma Aldrich, St. Louis, Missouri, United States) was added to each microcentrifuge tube. After incubation and washing procedure, CSF samples and solutions of standard protein (fully reduced and disulfide HMGB1) were added. Each sample was added to two tubes. Agarose conjugate, antibody and samples were incubated overnight in 4°C. After washing procedure each pellet was resuspended in 20 µl Laemmli sample buffer (Bio-Rad, Hercules, California, United States) or water and heated at 95°C for 5 min.

Gel electrophoresis

Samples resuspended in Laemmli sample buffer were further separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The 15 µl of prepared mixtures were loaded on the 12% Tris-glycine-SDS-PAGE gels. Separation was conducted at 200V for 40 min on a Mini-Protean Tetra Cell (Bio-Rad, Hercules, California, United States). The gels were stained with Coomasie Briliant Blue G250. The 9% acetic acid was used to wash out the background.

MALDI-TOF mass spectrometry (MS) fingerprinting

Stained spots were excised and digested with trypsin according to adapted Shevchenko et al. protocol [16]. Peptides were extracted using 35 µl of TA50 solvent. HCCA (alpha-Cyano-4-hydroxycinnamic acid) (Bruker Daltonics, Bremen, Germany) matrix solution was prepared by dissolving 1.4 mg of HCCA in mixture of 85% acetonitrile, 15% water, 0.1% TFA and 1 mM $NH_4H_2PO_4$. The 0.5 µl of the sample was spotted onto AnchorChip MALDI Target Plate (Bruker Daltonics, Bremen, Germany) and left do dry in the room temperature. Then the 0.5 µl of the matrix solution was spotted onto each sample spot and left do dry in the room temperature. Analysis was conducted using MALDI-TOF mass spectrometer (UltrafleXtreme, Bruker Daltonics, Bremen, Germany) in the reflectron mode in the mass range of 700–3500 Da. The mass spectra were externally calibrated using a standard peptide calibration mixture. Acquisition and spectra processing were performed by FlexControl 3.4 software (Bruker Daltonics, Bremen, Germany). Evaluation of the spectra was performed in FlexAnalysis 3.4

software (Bruker Daltonics, Bremen, Germany). Protein searches was conducted using BioTools 3.2 software (Bruker Daltonics, Bremen, Germany) SwissProt database and Mascot 2.4.1 search engine (Matrix Science, London, UK).

MALDI-ISD analysis

Samples resuspended in water after immunoprecipitation were analyzed using MALDI-ISD method, described earlier in MALDI TOP-DOWN SEQUENCING.

Results

MALDI top-down sequencing analysis

MALDI-ISD analysis for disulfide HMGB1 and fully reduced HMGB1 was performed. Top-down sequencing of the spectra was carried out using SwissProt database. From the reduced spectrum high score from the top-down sequencing search giving 'High mobility group protein B1 OS=Homo sapiens GN=HMGB1 PE = 1 SV = 3' was obtained (**Figure 2**). The N-terminus is missing the methionine. According to Swissprot there should be disulfide bond between cysteines at position 23 and 45 in disulfide HMGB1, which is not observed in fully reduced HMGB1 sample (**Figure 3**).

Immunoprecipitation and analysis of HMGB1 isolated from the cerebrospinal fluid samples

Protein spots on the SDS-PAGE gel appeared near mass 75 kDa and 20–25 kDa (CSF samples) and 25 kDa (HMGB1 standard solutions). Ig gamma protein (75 kDa) and keratin (25 kDa) were identified using MALDI-TOF MS fingerprinting, but HMGB1 was not fund. MALDI-ISD analysis of samples after immunoprecipitation procedure gave no results, there was no signal derived from protein.

Discussion

HMGB1 has been shown to play an important role as a mediator of inflammation. It is also involved in regulation of the immune response [17]. As HMGB1 seems to be implicated in diseases characterized by cell damage and death [18], it may serve as an indicator of SAH. Depending on appearance in different cellular compartments and redox status of HMGB1, its functions may vary. The aim of this study was identification of different HMGB1 redox forms using advanced mass spectrometry techniques. Obtained results confirm possibility of distinguishing between fully reduced and disulfide forms. In order to determine differences between redox forms with MALDI, disulfide

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  1 MGKGDPKKPR GKMSSYAFFV QTCREEHKKK HPDASVNFSE FSKKCSERWK
 51 TMSAKEKGKF EDMAKADKAR YEREMKTYIP PKGETKKKFK DPNAPKRPPS
101 AFFLFCSEYR PKIKGEHPGL SIGDVAKKLG EMWNNTAADD KQPYEKKAAK
151 LKEKYEKDIA AYRAKGKPDA AKKGVVKAEK SKKKKEEEED EEDEEDEEEE
201 EDEEDEDEEE DDDDE
```

Figure 2. Top-down sequencing search obtained from the fully reduced HMGB1 spectrum

Amino acid modifications

	Feature key	Position(s)		Description
T				{ECO:0000250|UniProtKB:P10103}.
T	MOD_RES	184	184	N6-acetyllysine.
T				{ECO:0000250|UniProtKB:P10103}.
T	MOD_RES	185	185	N6-acetyllysine.
T				{ECO:0000250|UniProtKB:P10103}.
T	DISULFID	23	45	In disulfide HMGB1.
T				{ECO:0000250|UniProtKB:P63159}.
T	VARIANT	11	11	G -> R (in gastric-carcinoma cell line).
T				{ECO:0000269|PubMed:9036861}.
T				/FTId=VAR_046451.
T	VARIANT	149	149	A -> E (in gastric-carcinoma cell line).

Figure 3. SwissProt database result, which confirmed that the cysteines at position 23 and 45 should be bonded in disulfide HMGB1 sample

HMGB1 and fully reduced HMGB1 were analyzed. MALDI-ISD analysis was performed to obtain fragmentation of the study proteins. Then top-down sequencing of the spectra was performed. In fully reduced HMGB1 cysteines at the positions 23 and 45 do not contain a disulfide bond. If the same sequence was assigned to disulfide HMGB1 sample, the sequence stops at position 21, which is right before the cysteine (**Figure 4**). However, there are quite some signals in the mass range > 2431 Da, which are unexplained. For a real verification of the disulfide link between positions 23 and 45 a signal at 5128 Da is needed, which then includes both oxidized cysteines.

In the next step of the study we made an attempt to isolate HMGB1 from the CSF of SAH patients by immunoprecipitation method, gel electrophoresis and trypsin digestion in order to identify its redox form using MALDI-TOF fingerprinting technique. Immunoprecipitation, a type of affinity purification, is one of the most widely used immunochemical techniques and it can be used to determine presence of proteins in biological material [19]. Following immunoprecipitation MALDI--ISD analysis was carried out. Despite the fact, that initial analysis of pure standard HMGB1 by MALDI-ISD was successful, after immunoprecipitation we were not able to identify HMGB1 in both CSF samples as well as standard. In the next step electrophoresis, digestion and MALDI-TOF MS fingerprinting analysis was carried out for remaining samples. Unfortunately, although keratin (25kDa) and Ig gamma protein (75kDa) were identified, we obtained no signal derived from HMGB1 in MALDI--TOF MS fingerprint method. In order to improve immunoprecipitation step we have used different antibody concentrations i.e. 1.25 µg/ml, 12.5 µg/ml and 100 µg/ml, but the results remained negative. Possible reasons of failure on immunoprecipitation step might be: degradation of HMGB1 antibody, inadequate time of incubation, inadequate washing solution or non-specific binding to Protein G. Nevertheless, since reduction of disulfide bonds is necessary step for tryptic digestion, it would be impossible to distinguish two forms of HMGB1 protein using this method. The preferred method of isolation of HMGB1 should allow for directly analysis using top-down MALDI-ISD strategy, without protein digestion or degradation.

Perspectives

As HMGB1 has been shown to play an important role in inflammation and cell damage processes, it may act as prognostic marker in the

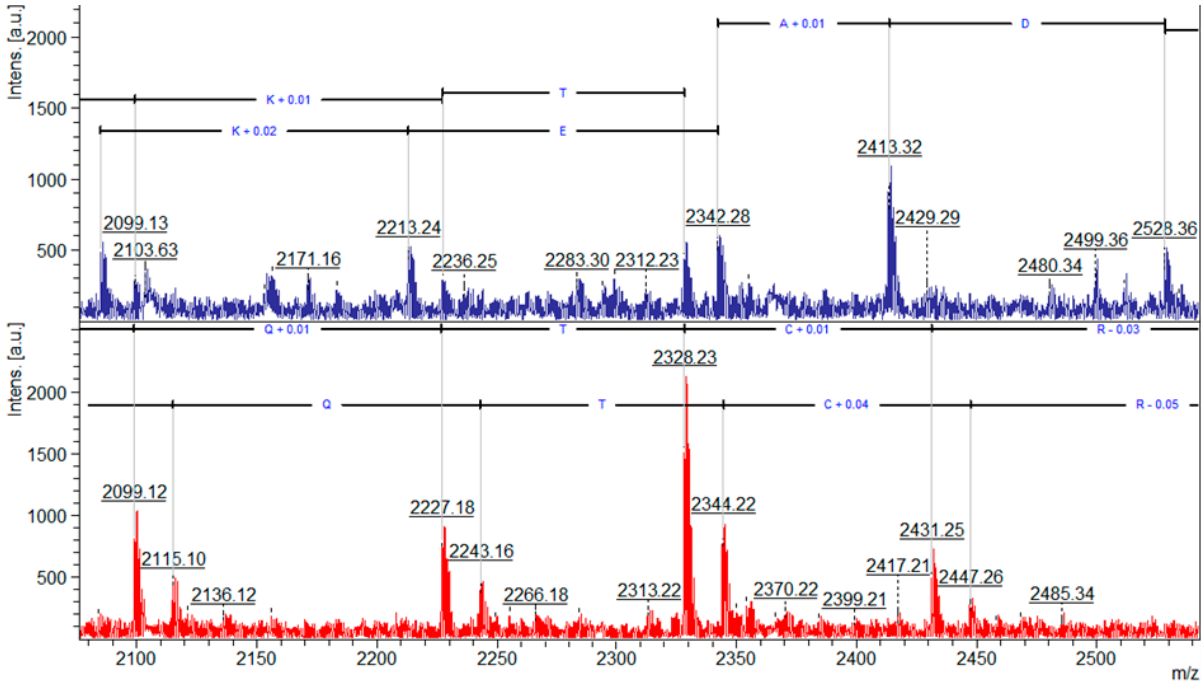

Figure 4. MALDI mass spectra of he fully reduced HMGB1 (blue) and disulfide HMGB1 (red). On the first spectrum (blue) fragmentation occurs before and beyond C22, which is not present on the second spectrum (red)

SAH patients. In further studies there is a need for optimization or development of new methods allowing isolation of HMGB1 from CSF and distinguishing between fully reduced and oxidized forms. We expect, that quantitative assay of different redox status forms would improve prognostic accuracy of HMGB1 as well as broaden our knowledge regarding pathophysiology of SAH.

Acknowledgements

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
This work was supported by a Diamond Grant No. DI2014 005044 from the Polish Ministry of Science and Higher Education (years: 2015, 2016, 2017, 2018).

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Acceptance for editing: 2019-07-30
Acceptance for publication: 2019-09-10

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REVIEW PAPER

DOI: https://doi.org/10.20883/jms.378

Oral tolerance induction and food allergy prevention

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ABSTRACT

This review aims to provide an overview of the issue of oral tolerance induction in early childhood and allergy prevention. We discuss changes in epidemiology of allergic diseases that have occurred over the last decades in the context of current knowledge about environmental factors affecting prevalence of these diseases. Also this article presents current data about causes of «hygiene hypothesis» expansion to «microflora hypothesis» as well as an immunological background of this process; describes how immune factors of cord blood and breast milk, maternal and infant's elimination diet, timing a solid food intake impact on immune system development and tolerance induction in early childhood. Current knowledge on issues of oral tolerance induction and allergy should induce update of allergy prevention recommendations in the nearest future.

Keywords: immune factors, cord blood, breast milk, microbiota, elimination diet.

Introduction

Immune tolerance is a state of "unresponsiveness" of the immune system to certain substances or tissue antigens that can potentially induce an immune response. Oral tolerance refers to a specific type of immune tolerance induced by orally ingested antigens such as food [1]. Therefore, it is often called oral tolerance to food antigens. It should be mentioned that gut immune system play a key role in oral tolerance induction. The earliest manifestation of failed oral tolerance induction is sensitization to food allergens. Subsequently, an allergen sensitization profile can expand and progress to clinical manifestation of food allergy and other allergic diseases as well [2]. The exact mechanisms involved in the development of oral tolerance as well as factors influencing this process are being actively studied and discussed, especially in terms of allergy prevention.

Since the 1980s the prevalence of allergic diseases, immune-mediated pathology such as diabetes mellitus type 1 and inflammatory bowel disease have been steadily increasing in developed countries while the incidence of infectious diseases have considerably decreased [3, 4]. WHO has declared an epidemic of noncommunicable diseases due to significant changes in environmental factors as a result of massive urbanization, changes in life style, human nutrition and medicine advancement as well [5, 6]. According to an EAACI prediction more than half of the European population will suffer from allergic diseases by 2025. Most of these diseases already manifest in childhood.

There are two major groups of factors, in particular genetic predisposition and environmental factors, which are crucial for allergic disease development. Obviously, we cannot explain significant changes in the epidemiology of allergies that have occurred over a relatively short period of time only by a genetic predisposition [3]. According to the latest data, about 10% of children who have developed allergic diseases in the first year of life don't have any relatives with allergic diseases and 20–30% of infants with allergy manifestations have first-degree relatives with allergic diseases [7]. The question is why children with negative family history of allergies develop allergic diseases and vice versa why in some cases genetic predisposition to allergy is not accompanied by disease manifestation or why it results in the allergic disease so early?

The role of gut microbiota

For the first time in 1989, D.P. Strachan drew special attention to the fact that allergic diseases were much less common in children who had several siblings and thus were more frequently exposed to infectious antigens. Based on this fact, the "hygiene hypothesis" explaining origin of allergic diseases was formulated by the scientist. Subsequently, this theory was confirmed by other researchers who described the lower incidence of allergic diseases in children living on farms during childhood (so-called "farm effect"), in kids having pets at home etc. [8, 9, 10].

About the same time (1986) T.R. Mosmann with colleagues described two types of T-helper cells (Th1 cells and Th2 cells) secreting different cytokines and therefore initiating various types of inflammatory response. It has been shown that Th2 cells play a key role in the development of allergic sensitization, while viral infection activates the Th1-mediated immune response and inhibits Th2 cytokine activity [11]. This data became the immunological basis of the «hygiene hypothesis». The rapid development of immunology has contributed to modification of an immunological concept of this hypothesis [12]. Of particular importance became the understanding of the gut mucosal immune system's role in immune system development and immune tolerance induction. Evidences provided by researchers over the past 10 years have promoted expansion of the «hygiene hypothesis». The crucial role of intestinal microbiota for immune tolerance induction and to support balance between Th1 and Th2 activities has been shown. This was the reason to rename a previous «hygiene hypothesis» of allergic diseases to «microflora hypothesis» [13, 14].

One of the much discussed issues is the effect of microbiota on immune tolerance induction and its relationship to allergic diseases manifestations, inflammatory bowel diseases, and diabetes mellitus type I [15, 16]. Experimental studies have demonstrated that germ-free mice cannot develop immune tolerance to food allergens [17, 18]. Clinical studies have revealed the link between early life gut dysbiosis and risk of allergic diseases in later life. For instance, children who developed asthma at school age had lower intestinal microbiota diversity during the first month of life [19]. Antibiotic use in early childhood has been shown to be associated with increasing risk of asthma development in early school age as well as inflammatory bowel disease and diabetes mellitus type I [20, 21, 22].

The first 1000 days of life are considered to be a critical period for the development of the individual gut microbiome composition that drives immune system maturation and has an effect on oral tolerance induction [23, 24]. T. Escherich (1857–1911) was a pioneer in studying the intestinal microbiota. His assumption about sterile intestine in utero and colonization by bacteria after birth has been refuted not so long ago [25]. Evidences that exposure to microbes can start in antenatal period have been provided thanks to achievements of molecular biology in the second half of the 20th century as well as recent progress in genome sequencing [26]. Recent studies have shown the presence of microorganisms in amniotic fluid and placenta even in the case of a healthy pregnancy [27]. This data have initiated discussion about changing the paradigm from «sterile womb» to «in utero colonization» hypotheses. [28]. Moreover, for the last decade researchers have been talking about microbial programming of health. For the first time, the hypothesis about fetal origins of many adult diseases, also known as fetal programming, was expressed by Barker D.J. in 1988 [29]. The term microbial programming, as a particular version of fetal pro-

gramming, appeared not so long ago. Despite this fact, there are a lot of studies confirmed this hypotheses [26, 23]. It has been shown that antibiotic use during pregnancy, nutritional habits of a pregnant woman, maternal age and her health condition influence on the maternal microbiome composition and this, in its turn, has an impact on the gut microbiota pattern in the offspring and consequently on the immune system maturation [30, 31]. It is worth mentioning here that human milk is considered to be an important source of initial bacterial colonization of the child's intestine after birth. Recent studies have declared that breast milk can be a source of ¼ commensal bacteria to the infant gut. Daily consumption of 800 ml of breast milk is accompanied by ingestion about 1×10^5–1×10^7 bacteria [32, 33]. It follows from the above that the process of microbial programming begins in antenatal period and continues after birth [34].

Effects of mother's immune factors in the antenatal period

In terms of fetal programming, studying the effect of mother's immune factors on the infant's immune system development during pregnancy has a particular interest. Previously it was believed that the placenta is a kind of barrier that protects fetus from mother's immune system and this way prevents fetus rejection. Recent studies have clearly shown that an impenetrable immunological barrier between mother and fetus is absent [28, 35]. It is already known about transplacental transfer of mother's IgG, cytokines as well as her immune cells (chimerism). These immune factors may interact with the developing fetal immune, that is to say, they might be involved in immune programming [36]. Spectrum of immunologically active components which receive fetus during pregnancy will depend on maternal health, in particular on her allergological status. There are some evidences that confirm the potential significance of events at this period of life for the development of allergies. For instance, researchers have described cases of food allergy development in adults for the first time after cord blood transplantation. This phenomenon was called «transplant-acquired food allergy» [37, 38]. Moreover, it has also been determined lower ratio Treg/Th2 and relatively lower concentration of Th1 associated cytokines (IF-γ) in cord blood of children born to mothers with allergic diseases [39, 40]. As indicated by other studies, children with a relatively lower number of regulatory T-cells as well as reduced ratio of Th1:Th2 in cord blood had a higher risk to develop an atopic dermatitis and to become sensitized to food allergens [41]. A cord blood IgE level as a predictor of atopy and allergic diseases has been studied for a long time; however, findings are conflicting. There are evidences that IgE levels in cord blood differs between mothers with and without atopy and correlates well with IgE levels in maternal blood. However, it is generally recognized that IgE do not cross the placenta barrier. In this regard, scientists have been discussing origin of IgE cord blood suggesting that a possible source of IgE might be its synthesis by the fetus as a manifestation of sensitization to allergens in utero or maternal-fetal transfer of these antibodies during labor. Recently it has become clear that not IgE but maternal cytokines can freely cross placenta barrier. Therefore, using cord blood IgE levels as a predictor of atopy in children is disputable [42, 44, 43]. Anti-inflammatory IL-10, as well as pro-Th2 cytokines such as IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) can easily cross the placenta barrier, thereby taking part in immune programming. Lower levels of Th1-associated chemokines (CXCL10, CXCL11) and higher levels of Th2-associated chemokines (CCL17 and CCL22) in cord blood have been reported to precede allergen sensitization in early childhood [45, 46, 43]. On the other hand, newly published data indicates a protective role of maternal allergen-specific IgG in relation to the development of allergen sensitization in children. There was shown that a higher titer of allergen-specific IgG in the mother's blood in third trimester as well as in cord blood and breast milk are associated with a lower incidence of sensitization to allergens in 5 year-old children [47, 48]. As is already known, food allergen intake by a healthy person mainly induces production of allergen-specific IgG promoting tolerance to these food antigens. The balance between allergen-specific IgE and IgG helps to determine if a person will develop symptoms. Therefore avoidance of allergenic foods during pregnancy should not be recom-

mended for healthy pregnant woman because it not to reduce the risk of sensitization of offspring to these foods and even opposite [49].

Immune factors of breast milk and timing of a solid food introduction

Breast milk is now being acknowledged not only as the best food for a child, but equally important as an exclusive source of immune-related components and biologically active substances. For instance, these components and substances include human milk oligosaccharides, cytokines, immunoglobulins, macrophages, T-lymphocytes (CD4 +, CD8 +, Treg), B-lymphocytes, stem cells, memory immune cells and many others. Immune factors of breast milk do not only «compensate» for immaturity of the infant's immune system, as previously suggested, but factors listed above can easily cross the intestinal barrier due to increased intestinal permeability for macromolecules and immunoglobulins in this period and can directly interact with immune cells in the gut mucosa. It has been shown that immunologic factors in human milk are able to train, modulate and promote the development of an infant's immune system as well as impact the intestinal colonization. It means immunologically active components in human milk can drive infant immune system maturation and affect oral tolerance induction [50, 51]. For example, predominant cytokines of breast milk such as transforming growth factor-beta (TGF-β) and IL-10 are involved in the induction of immune tolerance to food antigens as well as gut microbiota antigens. These cytokines are able to downregulate inflammatory response, are involved in switching from IgM to IgA in B lymphocytes, and suppress immunoglobulin E production; it is therefore expected to affect an oral tolerance induction [52, 53]. That is why a possible role of these breast milk cytokines in prevention or at least suppression of the onset of allergic diseases has been studying over recent years. According to some studies, the predominance of commensal microorganism in the mother's intestine result in a higher level of TGF-β and IL-10 in breast milk, and this in turn is associated with a lower risk of developing allergies in children. The longer TGF-β concentration in milk remains high, the more marked its protective effect against eczema in infants. Therefore, identifying factors that influence the level of TGF-β in breast milk could be a way to prevent food allergy [54, 53]. As it is well known, IL-4 and IL-5 are associated with the development of allergic inflammation; however, effect of these breast milk cytokines on oral tolerance induction is ambiguous [32, 55, 56]. Breast milk contains a wide range of cytokines (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-25, IFN-γ, TNFα, TSLP, and TGFβ etc.) but role of each one separately as well as its combinations in the tolerance induction and the infant immune system development are not completely understood and further studies will need to assess biological activity of cytokines taking into account its concentrations.

Furthermore there is conflicting data on the protective role of breastfeeding in relation to allergic sensitization and allergic diseases [57, 58, 59]. For instance, there are some studies that have demonstrated non-permanent or weak relationship between breastfeeding and risk reduction of allergy development, and in several cases the absence of such a link have even been declared [60, 61]. Scientists suggest that conflicting conclusions on this issue might have arisen because of different design of studies, in particular not the same duration of breastfeeding and diagnostic criteria for allergic diseases. However, according to results of current studies the composition of breast milk is considered to be the most important thing what was not taken into consideration in previous studies [62, 63]. It has shown, the human milk composition changes depending on duration of lactation (colostrum, transitional milk, mature milk), woman's health, maternal lifestyle and dietary habits, antibiotics use, and even geographic area of residence, socioeconomic factors [64, 65, 66]. Nevertheless, it is generally accepted, that immune-related components and biologically active substances of human breast milk influence the infant immune system development and, thus breastfeeding is capable of affecting a child's health in a long-term perspective. Researchers agreed that further studies should be provided to find out the relationship between the immune profile of breast milk and the risk of allergic diseases in children.

In addition to cytokines mentioned above, colostrum and mature milk are rich sources of immunoglobulins. The major immunoglobulin in

human milk is sIgA, concentrations of IgM and IgG are considerably lower. IgA is synthesized by B lymphocytes which migrate from the maternal intestinal *Lamina propria* to the mammary glands, known as «entero-mammary link». Antibodies specificity in breast milk reflects a range of antigen exposure in woman's intestine, therefore maternal elimination diet during lactation might influence a concentration of breast milk sIgA which specific to eliminated proteins [67]. This data have been confirmed by the recent study. It showed that mothers on elimination diet had lower concentration of cow's milk-specific IgA in human milk and it was associated with the development of cow's milk allergy in infants [68]. Once again it highlights the maternal elimination diet during lactation should not be recommended as a strategy for preventing child allergy.

Recommendations for infants to avoid allergenic foods to prevent allergic diseases were a mainstream in late 20th century. Recently scientists have been discussing «paradigm shift from allergen avoidance to tolerance induction by intake» [70, 69]. It was determined that the best timing of a solid food introduction is the period from 4 to 6 months. This period is already well known as a window of opportunity for tolerance induction to food allergens. Early introduction of food antigens may induce food tolerance faster than sensitization, while the later introduction of potentially allergenic foods may trigger an allergic response [70, 71]. Accordingly, in terms of tolerance induction the current data no longer supports delaying the introduction of potentially allergenic foods in infant's diet as well.

Conclusions

Thus, our knowledge about the process of oral tolerance and allergy development is constantly increasing. This new insight has brought new perspectives for allergy prevention. In terms of this issue specialists are focusing on induction of oral tolerance in early childhood and environmental controls including life style change. Obviously, clinical practice recommendations on allergy prevention for both high risk children and risk-free children should be revised taking into account newly available data, though further studies might be required.

Acknowledgements

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
There are no sources of funding to declare.

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Acceptance for editing: 2019-09-06
Acceptance for publication: 2019-09-10

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THOUSAND WORDS ABOUT...

DOI: https://doi.org/10.20883/jms.344

A thousand words about running fitness tests

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ABSTRACT

Running is undertaken for different reasons, including improvement or maintenance of health and fitness. Many tests are employed for the estimation of the fitness in runners. In this review, we describe five field tests (Cooper test, Conconi test, 6-Minute Walk Test, 20-meter Multistage Fitness Test, and Harvard Step Test) and one laboratory cardiopulmonary exercise test (CPET) on a treadmill. A properly selected fitness test may help to estimate or measure the maximal oxygen consumption (VO_2), thresholds for the aerobic and anaerobic metabolism, or restitution after the exercise. Such information is used for planning the training process, monitoring the progress of physical fitness or predicting the target distance or speed during competitions. In patients with cardiovascular or pulmonary diseases, this information may help to plan the intensity of daily activity or physical rehabilitation. Testing physical fitness is challenging, however when made appropriately, it delivers valuable physiological and clinical information.

Keywords: aerobic exercise, anaerobic exercise, cardiopulmonary exercise test, physical fitness, running, VO_2.

Introduction

Walking, running, cycling, swimming or physical labour are typical examples of physical activity, i.e. sustained body movement increasing energy expenditure [1, 2, 3]. If physical activity is undertaken to improve or maintain health and fitness, then it is called as an exercise [1, 2, 3]. Exercise is usually performed on a regular, repeated basis with a different frequency, duration and intensity. Exercise improves muscular strength (e.g. resistance training in powerlifting or bodybuilding), balance (e.g. tai chi) or flexibility (e.g. yoga) and is beneficial for the cardiovascular and respiratory physical fitness [3].

Physical fitness is defined as the ability to perform various aspects of sports, occupations and daily activities based on physical effort but without undue fatigue [3]. Different features of physical fitness may be quantified, for instance, aerobic fitness, muscular strength and endurance, flexibility, and body composition. In this review, we focus on selected tests applied to estimate physical fitness in runners.

For runners, cardiovascular endurance or aerobic fitness corresponds to the ability to jog or run continuously for an extended period without a lot of fatigue. The intensity of the running may range from low to high, and it depends mainly on the aerobic, i.e. requiring oxygen, metabolic processes generating energy [1, 5].

Many tests are employed for the estimation of the fitness in runners, from simple field tests (**Tables 1**, **2**, **3**) to a set of laboratory procedures and examinations with the cardiopulmonary exercise test (CPET) on a treadmill as the most advanced (**Table 4**) [4, 5]. The selection of a proper test should be based on the purpose of the research,

the target group (healthy vs sick people, amateurs vs elite runners), and the nature of the sporting effort, depending on its intensity and duration, reliability, costs and ease of use [1, 2, 4, 10].

It is possible to assess the effectiveness of the training, its intensity and applied loads (e.g. total mileage) by comparing the results of several tests performed at different phases of preparation for the competition. Adaptation to the repeated training can be assessed in runners in top sports laboratories that use specialised and expensive equipment, for example for the CPE) to measure the maximal muscle oxygen consumption (VO_{2max}) [5]. However, such tests are not necessary for all runners, and the majority of them can be examined in the gymnasium or track and field through the use of simple, functional field tests [1, 2, 4]. For some of such tests, different formulas allowing the estimation of VO_{2max} have been developed.

Field tests are used for people at the different level of physical fitness, from low to high, in enthusiasts of recreational running, jogging, Nordic walking, amateur and elite runners [1, 2, 4]. The field tests usually do not require specialised equipment. The main rule is to perform the test on a track or route with a precisely measured distance with the use of a stop- or sport-watch or to measure the distance covered in a specific time [4]. For many years, the pulse rate has been measured manually by counting the number of pulsation over carotid or radial artery. In recent years, it has also become possible to quantify heart rate more precisely, during and after the fitness test, even without interrupting physical activity [4, 6]. The heart rate can be measured by so-called heart rate monitors which are strips placed on the chest. Another solution with increasing popularity is the use of wearable devices, smart and sports watches which have special infrared sensors measuring the capillary pulsation that usually equals to the heart rate [6]. For some tests performed in specific populations, e.g. patients with pulmonary or cardiac diseases, equipment to measure blood oxygen saturation (sO_2) is required for example during the CPET or the 6-minute walk test [5, 7].

Field tests

Each type of field test has its purpose. For the Walking/Running Tests — subjects are either walking or running as fast as possible for a specific time (e.g. exactly 6 or 12 minutes) or a set distance (e.g. 400 m, 1 km, 1 mile, 5 km etc.) [1, 2, 4, 7–21]. In the Maximal Aerobic Tests, the exercise is continued until exhaustion, which means it must cover the aerobic capacity and reach the anaerobic level [1, 2, 4, 8, 10]. In case of the Intermittent or Interval Tests, also performed until exhaustion, the consecutive stages with continuous running are separated by periods of either rest or substantial slowing down of the running pace [4, 10, 16–18]. Finally, in Step Tests, the principal element is repeated stepping up and down on the platform at a given rate for either a certain time or until exhaustion [1, 2, 4, 19–21].

Table 1 lists different types of field tests performed in runners with some examples of specific tests. **Table 2** summarises the methodology, types of subjects, advantages and disadvantages of the

Table 1. List of the most popular field tests for runners [1, 2, 4, 9–11, 13, 16, 19]

Types of tests	Examples of tests
Running/Walking Tests	Cooper 12 minutes RunTest
	1-km Run
	Conconi Test
	6-Minute Walk Test
	Rockport Walk Test
Maximal Aerobic Tests	20-meter Multistage Fitness Test (MSFT) (shuttle run test, beep test)
	Yo-Yo Endurance Test
	Maximal Oxygen Consumption Test (VO_{2max})
Intermittent (Interval) Tests	Interval Shuttle Run Test (ISRT)
	The Yo-Yo Intermittent Tests
	Gacon Test (Running 45''/15'')
Step Tests	Harvard Step Test
	Step in Place

most popular field tests for runners. **Table 3** explains how the most popular five different field tests can be performed. Parameters measured during the selected field tests are described in **Table 4**.

The Cooper 12-minute Run Test is the easiest way to measure physical performance in healthy people. The distance covered during the test is compared with the data included in the special table, taking into account the sex and age of the examined person [8]. The Cooper test can be used to: measure the actual physical performance and predict its potential in response to training, measure improvement related to training and even as a way for athlete's motivation [4, 9, 10].

The Conconi test is applied to estimate individual anaerobic threshold during a continuous running with gradually increasing speed. The test results are shown as a value of heart rate and speed above which running is continued in anaerobic conditions. By subtracting 20 beats from the anaerobic threshold for heart rate, the aerobic threshold can also be estimated [4].

The six-minute Walk Test (6MWT) is an adaptation of the Cooper's 12-minute run test for

Table 2. Basic technical requirements, rules of scoring, subjects, advantages and disadvantages of the most popular field tests for runners [1-4, 7, 18].

Test	Equipment	Scoring	For Whom	Advantage	Disadvantage
Cooper 12-minute run test	Running, optimally, on a 400-m track, marks every 50 meters, stop-watch, heart rate monitoring device.	Individual endurance estimated by specially developed tables - Cooper test norm tables, specific for age and gender. Different criteria for professional athletes.	Professional & amateur runners. Can be done as a walking test for unable to run.	Simple and cheap, possibility to simultaneously test a large number of people. Very well studied in athletes.	Practice required. Motivation may affect the result.
Conconi Test	Running, optimally, a 400-m track, marks every 200 meters, stop-watch, heart rate monitoring device.	Anaerobic threshold determined from the graph showing the relation between running speed and heart rate. The deflection point on this graph (flattening of the heart rate after linear increase) indicates the athlete's anaerobic threshold in beats/ minute.	For endurance sports athletes who can perform maximal effort. Mainly for the elite and top amateur runners.	One of the most precise field test to determine the anaerobic threshold in natural conditions.	An experienced coach or trainer. Practice required. Special form, application or formula in excel or other software allowing to make X-Y graphs.
Six-minute Walk Test	Stopwatch, measuring tape or a flat corridor of at least 30-m length, chairs for resting.	The distance covered during the 6-minute walk.	Seniors who are unable to participate in traditional fitness tests. Patients with cardiac and pulmonary disease.	Simple and cheap, very well studied in clinical conditions.	Testing one person at a time. Not designed for very fit people. Running is forbidden, but brisk walking is enhanced.
20-meter Multistage Fitness Test (MSFT)	Flat surface, marking cones, measuring tape, beep test audio, music player, recording sheets.	The completed level and number of the twenty-meter shuttles.	Sports groups, children & adolescents, healthy people; adults with quite high physical capacity.	Simple and cheap, possibility to simultaneously test a large number of people. Attractive can be performed in the form of the competitive game. Very well studied in target populations.	The necessity of using electronic equipment and anti-slippery surface. Type of shoes has a large impact on the result.
Harvard Step Test	step or a gym bench of 45 to 50.8 cm height, metronome, stopwatch, heart rate monitoring device.	The Fitness Index score is determined by measuring pulse or heart rate 1, 2 and 3 minutes after completion of the test.	Useful in endurance sports, better for an amateur than professional athletes.	Self – administrated, simple and cheap, minimal equipment is required. A few people can be tested at the same time.	Individual biomechanical characteristics modify the results - easier for taller people.

people who are unable to run. In contrast to the Cooper's test, there is no warming-up and running is forbidden (for those who can run, other tests, e.g. Cooper's test, should be applied). It is used to evaluate the functional fitness of older people and patients with chronic disorders of the respiratory tract and lungs (for example chronic obturatory pulmonary disease) or the heart (heart failure of different causes) [7, 11–14].

The 20-meter Multistage Fitness Test (MSFT) is usually performed in young participants (children, adolescents), fit people and athletes. There are 23 levels with several repetitions of a series of 20-meter shuttles. The duration of shuttles during the first level corresponds to the speed is 8.5 km/hour (or tempo of 7 minutes and 4 seconds for 1 km) and increases by 0.5 km/hour at every next level [4]. Each level lasts about 1 minute, and there is an increasing number of the shuttles from 8 shuttles in the first level to 16 shuttles in the last level (due to shorter duration of each shuttle with increasing running speed) [4, 16–18].

Harvard Step Test is a test based on the analysis of the heart rate recovery after the repetitive stepping up and down for 5 minutes. It is a good tool to measure fitness efficiency and

Table 3. Summary of instructions on "How to do" selected types of field tests for runners [1–4, 7, 18]

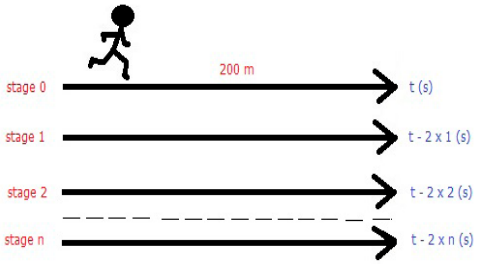
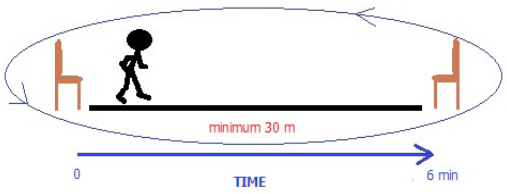
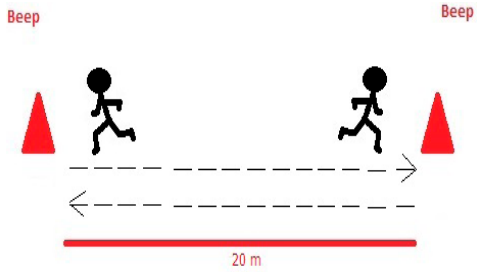
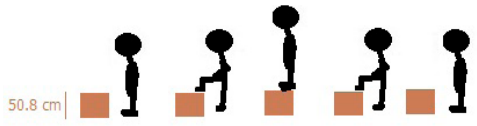
Diagram how to do the test	Description of the test
	*The Cooper test* Warming up for 10 minutes before the test. After the command "GO", the stop-watch is started and the subject commences the test by running for 12 minutes as fast as possible. The assistant informs the subject of the remaining time of running. After the 12 minutes, the test is stopped and the covered distance measured.
	*The Conconi test on a 400-m Track* Warming up for 5 to 10 minutes before the test. After the command "GO", the stop-watch is started and the subject runs gradually increasing the running speed every 200 meters, for example by cutting the duration for each 200-m by 2 seconds. The assistant informs about the duration of the last 200 meters. The increase in speed is maintained until the athlete is unable to accelerate. The starting speed and acceleration between the 200-m stages should be individually adjusted. The diagram on the left shows that the duration of each 200 m is shortened by 2 seconds. Usually, the covered distance is between 2.5 km and 4 km. The measured heart rate is plotted against the speed on the X-Y graph for the determination of the anaerobic threshold.
	*The six-minute walk test* Flat, straight corridor of at least 30-meter length with turnaround points. The patient rests in a chair for at least 10 minutes before the test. Heart rate and pulse oxygen saturation (sO_2) should be monitored continuously. Instruct the patient about walking as fast as possible for 6 minutes, but not running. Encouragements should be provided every one minute. At the end of the test, mark the spot where the patient stopped on the floor. Measure the pulse rate and SpO2 if possible. Calculate the distance walked.
	*The Beep Test* The participant is familiarised with the rules and warms up. Then, the athlete should: run after the beep from the first to the second marker and get there before the next beep sounds; wait for the next beep to run back to the first marker; This sequence is repeated for each shuttle until the participant is unable to keep up with the beeps. A warning is given to the participant for the first fail when the marker line is not reached. The second warning eliminates the participant from the test. The level and number of 20-meter shuttles reached before exclusion is used as the test's score.
	*The Harvard Step Test* The subject steps up and down on the platform at a rate of 30 steps per minute for 5 minutes or until exhaustion (an inability to maintain the stepping rate for 15 seconds). On completion of the test, the subject immediately sits down. Heart rate is counted after 1, 2 and 3 after finishing the test.

Table 4. Parameters measured in the most common field tests and their potential correlation with VO_2

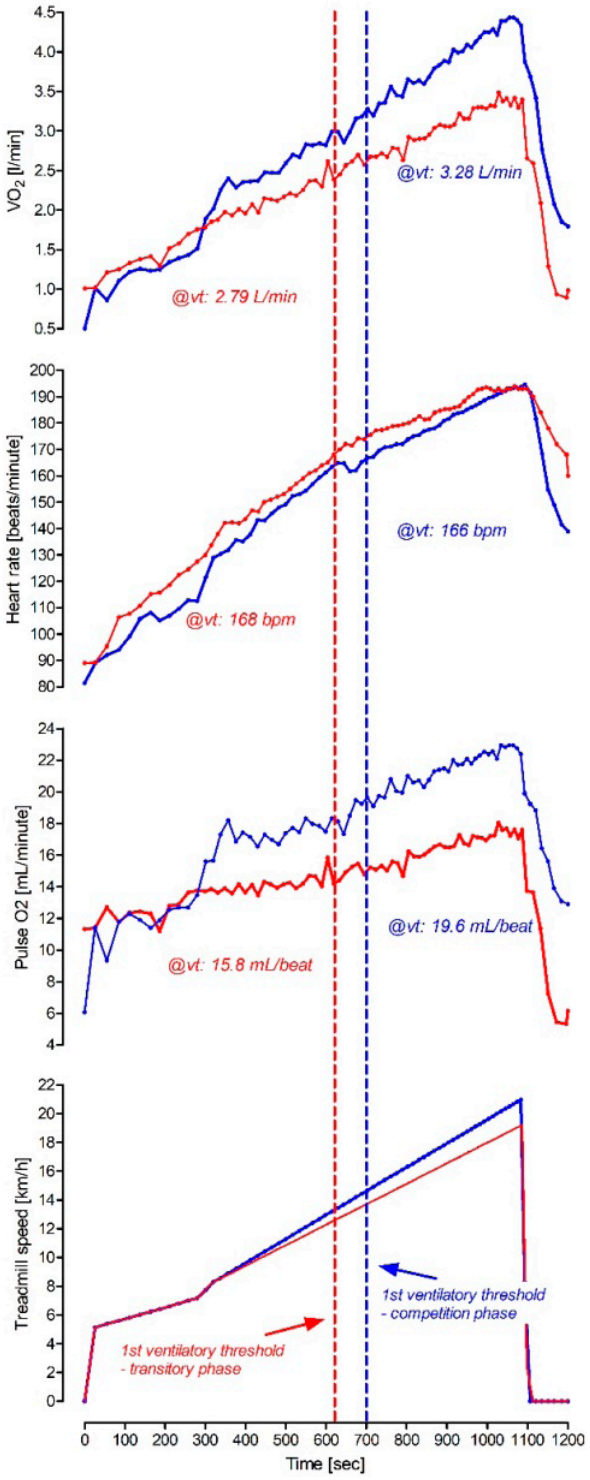
Test name	Measured parameters	Correlation with VO_2
Cooper 12 minutes Run Test	Distance covered by running in 12 minutes; Estimated VO_2max (in ml/kg/min): eVO_{2max} = (distance - 504.9)/44.73	High correlation with VO_{2max}, r = 0.90 [10].
Conconi Test	Continuous recording of heart rate. Averaged values of heart rate for each run 200-m is plotted against the running speed. The deflection point of the running speed – heart rate relationship is considered as the anaerobic threshold.	It is not designed for the VO2 estimation. The main goal is to estimate the anaerobic threshold. However, there are sparse data on the strength of the relationship between this test and the anaerobic threshold from weak to very strong [22].
6 Minute Walk Test	Distance covered by brisk walking in 6 minutes;	This test elicits peak VO_2 similar to that observed during the CPET test but at the lower ventilatory requirements. Weak to moderate strength correlation (r = 0.4–0.8) with VO2 in patients with lung diseases. May co-predict 79–82% of measured VO_{2max} in women and men. An independent predictor of mortality, morbidity and risk of hospitalisation in patients with chronic respiratory diseases, heart failure [7, 13–15].
20-meter Multistage Fitness Test (MSFT)	Level of the test Estimated VO_2max	High correlation with the actual VO_{2max} for adults r = 0.95–0.975 and for children and adolescents r = 0.89 [16–18]. The Beep VO_{2max} Calculator estimates the VO_{2max} score equivalents for each level of the test.
Harvard Step Test	The fitness index score ***Fitness Index*** (short form) = (100 x test duration in seconds) divided by (5.5 x pulse count between 1 and 1.5 minutes). ***Fitness Index*** (long form) = (100 x test duration in seconds) divided by (2 x sum of heartbeats in the recovery periods).	Moderate (r = 0.66 – 0.72) to very strong correlation (r = 0.92) with VO_{2max} in adults of low fitness level [4, 18, 23, 24].

participant's ability to recovery after a vigorous exercise. The more quickly heart rate returns to the pre-test level, the more fit is the person or the better is restitution after the exercise [19–21].

Cardio-Pulmonary Exercise Test

The Cardio-Pulmonary Exercise Test (CPET) is a non-invasive method used for assessing physical performance [1, 2, 5, 10, 25, 26]. The CPET test allows examining the function of the cardiovascular and respiratory systems under strictly defined conditions of metabolic stress, which is caused by a physical effort with a gradually increasing load over time. The CPET helps to estimate approximate thresholds between aerobic (1st ventilatory threshold or anaerobic threshold), mixed (aerobic-anaerobic), and anaerobic metabolism (2nd ventilatory threshold or the respiratory compensation point — RCP). A set of parameters is continuously measured (**Table 5**) through either heart rate monitor or ECG (heart rate), a sensor for sO2, and analysis of partial concertation of oxygen and carbon dioxide (CO_2) in the breathing air as well as tidal volume and respiratory rate. Among the measured parameters are muscle oxygen consumption (VO_2), production of CO_2 (VCO_2) or pulseO_2. This test is regularly employed in elite runners, particularly medium and long-distance runners, triathlonists. In modern coaching of runners, results of CPET are used to determine the training loads to increase the effectiveness of the training [10, 25, 26]. The CPET is also used in clinical practice in patients with advanced pulmonary and heart diseases (e.g. potential candidates for heart transplantation) or to diagnose undetermined dyspnoea. The test can be made on a treadmill or cycle ergometer [5].

In summary, physical fitness has many features and can be assessed by dozens of tests. Such tests should be carefully selected, according to specific reasons for their performance and the features of physical fitness to be tested. The decision about the test choice should also be based on the studied population and the availability of the test. Some tests are better to test endurance; some examine the aerobic or anaerobic metabolism and other post-exercise restitution. The field test for runners cover most of these issues



Abbreviations: @vt — at the ventilatory threshold; O_2 — oxygen; VO_2 — oxygen consumption; pulse O_2 — oxygen pulse defined as a ration of VO_2 to heart rate — it corresponds to the left ventricular stroke volume (depends on myocardial contractility) and arterio-venous oxygen difference. Usually, the arterio-venous difference in oxygen concentration does not change a lot during exercise

Figure 1. Sample results of two cardiopulmonary-exercise tests performed in the same 38-year-old male elite long-distance runner during the transitory phase (red curves and descriptions) and the competition phase (blue curves and descriptions) separated by ten weeks of systematic training. During the 10-week training period, the runner gradual increased his running load from 80 to 200 km a week. At this time, his maximal normalised VO2 increased from 57.1 to 71.4 mL/min/kg. There are visible changes in the presented parameters — the training caused a significant improvement in VO_2 and pulse O_2 curves with the lowering of the heart rate curve. Both CPET tests were performed using the individualised ramp protocol, after the 5-minute warm-up, the angle to the treadmill elevation changed from 0 to 1%. The speed of the treadmill started at 8 km/h, and then it changed 0.1 km/h every 7 seconds during the transitory phase and every 6 seconds during the competition phase

Table 5. Parameters measured during the cardiopulmonary exercise test using a treadmill [5, 25, 26]

Parameter	Description
Bf	Breathing frequency (breaths/minute)
HR	heart rate (beats/minute)
MET	metabolic equivalent; expressing work done as a multiple of resting energy expenditure. 1 MET equals 3.5 ml/min/kg of VO_2
O_2Pulse	Oxygen pulse, i.e. the ratio of VO_2 to heart rate. This parameter is proportional to the arterial-venous difference in oxygen pressure and stroke volume
$PETCO_2$	end-tidal partial pressure of carbon dioxide (mmHg)
$PETO_2$	end-tidal partial oxygen pressure (mmHg)
RER	respiratory exchange ratio
VCO_2	carbon dioxide production (l/min)
VE	minute ventilation (l/min)
VE/VCO_2	minute ventilation/carbon dioxide production
VE/VO_2	minute ventilation/oxygen consumption
VO_2	Oxygen consumption (l/min)
VO_2/kg	Oxygen consumption normalised to body weight (ml/min/kg)
Vt	Tidal volume (ml)

— but for patients with chronic heart or pulmonary diseases, they may not be best fitted. In all cases, i.e. elite athletes, amateur runners and in patients with different diseases, the most accurate and detailed is the CPET. However, this test should be performed with the use of an individualised exercise protocol that takes into consideration the potential level of fitness and the maximal walking or running speed sustainable by the examined person for a couple of minutes.

Acknowledgements

Conflict of interest statement
MF receives a scholarship within the Implementation Doctorates Program from the funds of the Ministry of Science and Higher Education, Warsaw, Poland. Project number 0084/DW/2018/02.

Funding sources
This review is a part of the project 0084/DW/2018/02 realised within the "Implementation Doctorates Programme" from the funds the funds of the Ministry of Science and Higher Education, Warsaw, Poland.

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Acceptance for editing: 2019-08-15
Acceptance for publication: 2019-09-10

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DOI: https://doi.org/10.20883/jms.245

Genetics in familial hypercholesterolaemia — from genetic research to new guidelines

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ABSTRACT

Familial Hypercholesterolaemia (FH) is genetic disorder touching up to 1 to 250 people, increasing the risk of atherosclerotic cardiovascular disease risk and early death by 3–13 times. The majority of mutations are autosomal dominant among 3 genes related to cholesterole metabolism: LDL-receptor (LDLR), apolipoprotein B (APOB) or proprotein convertase subtilisin/kexin type 9 (PCSK9). It comprises 60% of reported cases, which still is not at satisfactory level. This article summarizes new research in the field of FH and points out new therapeutic methods — PCSK9 inhibitors as advised in new European Society of Cardiology guidelines od dyslipidaemias.

Keywords: familial hypercholesterolaemia, PCSK9 inhibitors, evolocumab, alirocumab, dyslipidaemias.

Introduction

Familial hypercholesterolaemia (FH) is the genetic disorder touching 1 in 500 to, where the founder effect can be observed, even 1 in 250 people. Patients with this disorder have 3–13 times greater risk for developing premature atherosclerotic cardiovascular disease (ASVD) comparing to non-FH patients with normal blood lipid concentrations. It results in higher risk for sudden cardiac death, myocardial infarctions and ischemic heart disease events (i.e. frequent coronaroplasty), significantly reducing the life-expectancy of these patients [1].

Majority FH is linked to autosomal dominant inheritance of mutations in either LDL-receptor (LDLR), apolipoprotein B (APOB) or proprotein convertase subtilisin/kexin type 9 (PCSK9) and other loci — 60% of FH patients is mutation-positive. Also single nucleotide polymorphism of LDL-cholesterole (LDL-C) was found in FH patients mutation-negative suggesting the 88% of them has polygenic basis [2]. LDLR is responsible for the LDL-C blood concentration by regulating LDL-C uptake by cells and until now has more than 1000 mutations reported among FH patients, being most described pathogenetic genetic cause of this disease. APOB enables binding between LDL-C molecules and LDLR. PCSK9 is an enzyme responsible for degradation in LDLR. FH is linked to cardiovascular events in earlier age, needs early diagnosis and treatment, if possible- in the childhood to extend life-expectancy to the FH patients of overall population [3]. To do so, Dutch Lipid Clinic Network (DLCN) criteria are used, which include: pedigree analysis for premature cardiovascular events in 1 degree relatives, cholesterol concentration, medical history of an individual and symptoms [4].

Although FH genetics and inheritance is well described there are some new data reported in recent years. Also it is worth pointing out that in already finished genetic studies only around 60% of patients have investigated mutations, which leaves a lot of space for future projects. Novel genetic variants were studied in Saudi Arabia in 12 FH-linked genes (LDLR, APOB, PCSK9, Abca1, Apoa2, Apoc3, Apon2, Arh, Ldlrap1, Apoc2, ApoE, and Lpl) that have been implicated in the homozygous phenotype of a proband pedigree to identify candidate variants by next generation sequencing. The investigators questioned classical genetic sequencing methods as ineffective due to multiplicity of loci in genome linked with lipid metabolism genes. This study confirmed only LDLR, APOB and PCSK9 genes were associated with FH, new location was found for heterozygous variant, the rest of 7 validated viariants was not linked with FH [5].

Next-generation targeting was used also in Swedish study of 77 FH patients investigating LDLR, APOB and PCSK9 genes. The result obtained remains satisfactory — 65% of probants had the studied variants, leading to the conclusion that next-generation sequencing together with DLCN criteria might be helpful tool in diagnosis for FH [6].

FH cohort studies are rare, because still FH is underdiagnosed. Spanish registry included 2938 patients belonging to 775 families with positive genetic testing for the FH. The multicenter research started in 2004 dividing genetic variants according to American College of Medical Genetics and genomics: total 194 variants, 65 were functionally linked with cause of FH, 111 — pathogenic and 59 likely pathogenic, giving 88% of studied variants. Interesting to note that 11% of the rest were of unknown significance leaving big space for further investigation. Patients were divided into groups of null alleles (911 pts), defective alleles (1259 pts) and non-determined alleles (NDA, 473 pts). Null alleles patients presented higher risk score according to their blood tests results, needing higher doses of statins or other treatment [7].

The genetic tests resulted in new treatment method. As recommended by European Society of Cardiology in 2016 in the Guidelines on Dyslipidaemias appeared not only statins, bile acid sequestrants, cholesterol absorption inhibitors and nicotinic acid, but also PCSK9 inhibitors — evolocumab and alirocumab [8]. Both are antibodies that combine with hepatocytes to increase the breakdown of LDL-C. They are injectable drugs taken twice a month together with cholesterol level lowering drugs when the patient's LDL--C levels are not satisfactory even though all the medications are at maximal dosage. FH patients are strong candidates for target group for PSK9 inhibitors. Evolocumab yields 60% lowering of LDL-C, giving the patients better chance to survive longer [9]. New drugs are still developed, so there is still a chance that genetics will not kill those patients anymore.

Acknowledgements

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
There are no sources of funding to declare.

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Acceptance for editing: 2019-08-10
Acceptance for publication: 2019-09-30

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THOUSAND WORDS ABOUT...

DOI: https://doi.org/10.20883/medical.391

A thousand words about the challenges of photodynamic therapy

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ABSTRACT

The outbreak of interest in photodynamic therapy (PDT) at the end of last century to treat cancer and other diseases was based on the promise of localised treatment, cheaper therapy and fast ablation of the treated organ. One of the most attractive features of PDT is that it can evade cancer's resistance to photosensitisers. PDT for cancer therapy depends on the absorption of a photosensitiser within the malignant tissue. The photosensitising drug is then activated by light (usually from a laser) and the active drug destroys the targeted tissue. However, one must consider that this is a complex mechanism involving many factors such as the diverse light and oxygen distribution in the treated organs, which has mitigated application of this technique in clinical practice. PDT is not a simple treatment that can be done by eyeballing; it requires precise planning that can be done with the help of complex computer programs. Computer simulation of PDT to optimise treatment depends heavily on intense calculations in all steps of the procedure, and desktop computers are only now sufficiently powerful to assist physicians during therapy in real-time. In this mini-review, the challenges of photodynamic therapy are described, and possible to solutions to overcome these are presented.

Keywords: photodynamic therapy, cancer, computer simulation.

The explosion of interest in photodynamic therapy (PDT) at the end of the last century to treat cancer and other diseases was based on the promise of localised treatment, cheaper therapy and fast ablation of the treated organ. Many authors reported successful application of PDT techniques *in vitro* [1–6], but *in vivo*, PDT has been investigated to treat different diseases mostly when light can be easily delivered [1, 7–9]. Currently PDT is used mainly in dermatology, and in the treatment of glioblastoma and mesothelioma, and some efficacy of this modality has been demonstrated [10]. One of the most attractive features of PDT is that it can evade cancer resistance to photosensitisers [10–13]. However, one must consider that this is a complex mechanism involving many factors such as the diverse light and oxygen distribution in the treated organs, which has mitigated application of this technique in clinical practice [14].

PDT for cancer depends on the absorption of a photosensitiser within the malignant tissue. The photosensitising drug is then activated by light (usually from a laser) and the active drug destroys the targeted tissue. There are four major components of photodynamic therapy: light, photosensitiser, oxygen, and the tissue characteristics of the treated organ.

Light

The light used for PDT is usually in the wavelength range of around 600–800 nm, and is called

the therapeutic window. Light in this range has the right energy level (>1.5 eV) to excite the photosensitiser, and is a wavelength that ensures sufficient penetration into the tissue [15]. Higher wavelengths offer better tissue penetration [16, 17], thus, some authors define a therapeutic window up to 1000 nm, however some light absorption by water in the range above 900 nm must be considered as it will limit tissue penetration to some extent [18].

Photosensitiser

Quinones and porphyrin derivatives which absorb light in the therapeutic window are most frequently used in photodynamic therapy [19]. The photosensitiser should preferentially accumulate in cancerous tissue (at least twice as much as in the surrounding tissue) [20]. Sometimes, to increase the concentration in the PDT target, the photosensitisers are conjugated with an antibody specific to the cancer cells to increase drug build-up [16]. Upon being irradiated with a low-power light and absorbing photons, the sensitised photosensitisers in the presence of oxygen produce several radicals and reactive oxygen species (ROS). Among them, singlet oxygen is the primary active specimen causing necrosis in the treated organ [19, 21].

Tissue characteristics

The two parameters of greatest importance in photodynamic therapy are the attenuation coefficient (absorption and scatter coefficient of light within tissue), and the critical fluence (minimum energy of light needed to kill cancerous tissue). Both depend on the concentration of the active drug within the tissue. The distribution of light energy fluence φ_l in J cm^{-2}, around a cylindrical fibre, placed in a highly scattering medium, such as cancer tissue, is based on the diffusion equation:

$$\varphi_l = \varphi_o \exp(-r\,\mu_{eff})$$

where: φ_o is the energy fluence at the light source, μ_{eff} is the effective attenuation coefficient in cm^{-1} that describes the absorbing and scattering properties of the tissue, and r is the distance from the delivery fibre in cm [17]. This equation is sufficient to calculate the extent of treatment, as can be seen on **Figure 1**.

Computer simulation

This represents the ideal situation. In reality, light distribution from light diffusers is not cylindrical and the tumour is not cylindrical in shape either. This requires the three-dimensional shape of the tumour or treated organ to be obtained. For example, in the case of prostate cancer, the preferred treatment is to ablate the entire organ [22]. In this case, a three-dimensional model can be constructed from two-dimensional ultrasound images, including the positions of inserted light sources, as can be seen in **Figure 2**. Moreover, having multi-sensor probes, the spatial distribution of the optical properties can be measured.

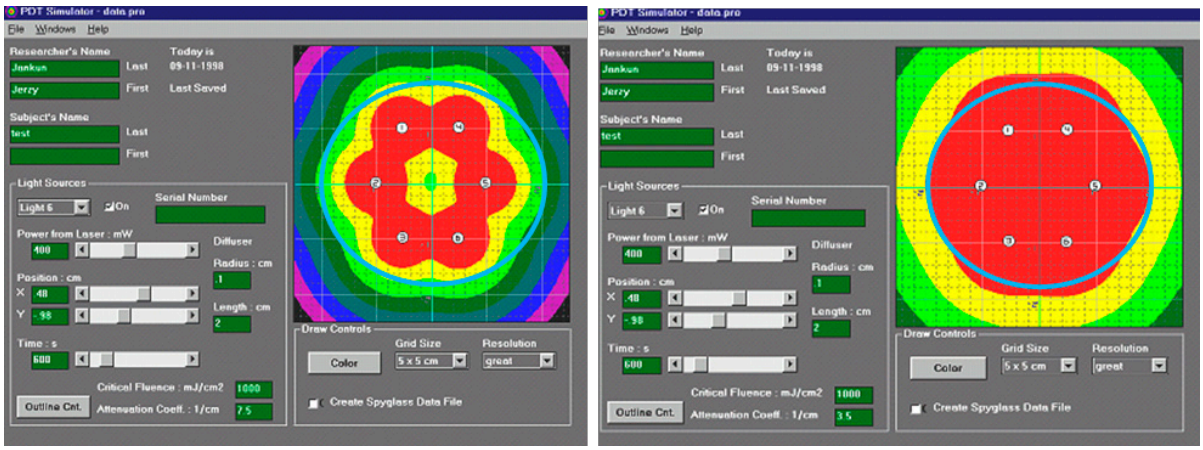

Figure 1. PDT computer simulation. Cross-section of imaginary cancerous tumour with (blue line) 6 light diffusers inserted into the tissue. The red areas represent the minimum light fluence required to effectively activate the drug and consequently ablate the tissue. Each successive colour band denotes isosurfaces of 10 times lower light fluency. *Left picture*: Not all cancerous tissue was ablated as the yellow colour inside the tumour indicates 10 times lower fluence than is needed for therapeutic ablation. *Right picture*: The red area filled the entire tumour cross-section when the critical fluence was smaller, or the time of treatment or light power was increased [20]

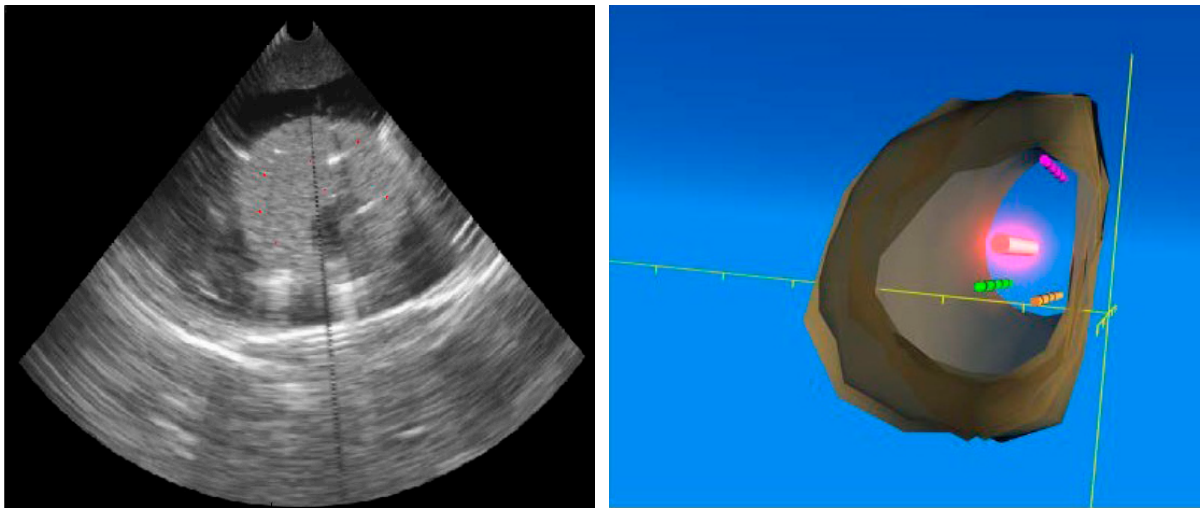

Figure 2. *Left picture*: Transverse ultrasound view of the prostate model showing the location of the laser diffuser and multi-sensor probes for fluence rate monitoring (white marks). *Right picture*: Model of the prostate based on transverse ultrasound images. For clarity, the base and apex of the model were "cut". The glowing red rod represents the light diffuser, and the three other rods represent multi-sensor probes for fluence rate monitoring

Uneven distribution of the photosensitiser within the treated organ is an additional complication of PDT. For example, we analysed the spatial distribution of a photosensitiser (tin etiopurpurin dichloride (SnET2) encapsulated in liposomes) in a canine prostate and found that its concentration varies between 1 and 2.5 μg of SnET2 per gram of tissue (**Figure 3**) [17].

The fluctuations of the photosensitiser within the treated organ can result in variation of the therapeutic PDT effects [21, 23]. Thus this parameter can be quantified by measuring the absorption of light between the light diffuser and segments of multi-sensor probes in a wavelength characteristic of the individual photosensitiser. The tissue's optical properties are influenced not only by the concentration of photosensitiser, but also by oxygenation of the blood, which can be measured in a similar way. Moreover, the amount of oxygen changes during photodynamic therapy, and the amount of photosensitiser changes as a result of consuming oxygen and the process of photobleaching. To account for these interactions, in addition to all of the parameters described above, a dynamic model of the photodynamic process is required to predict therapeutic tissue damage [15].

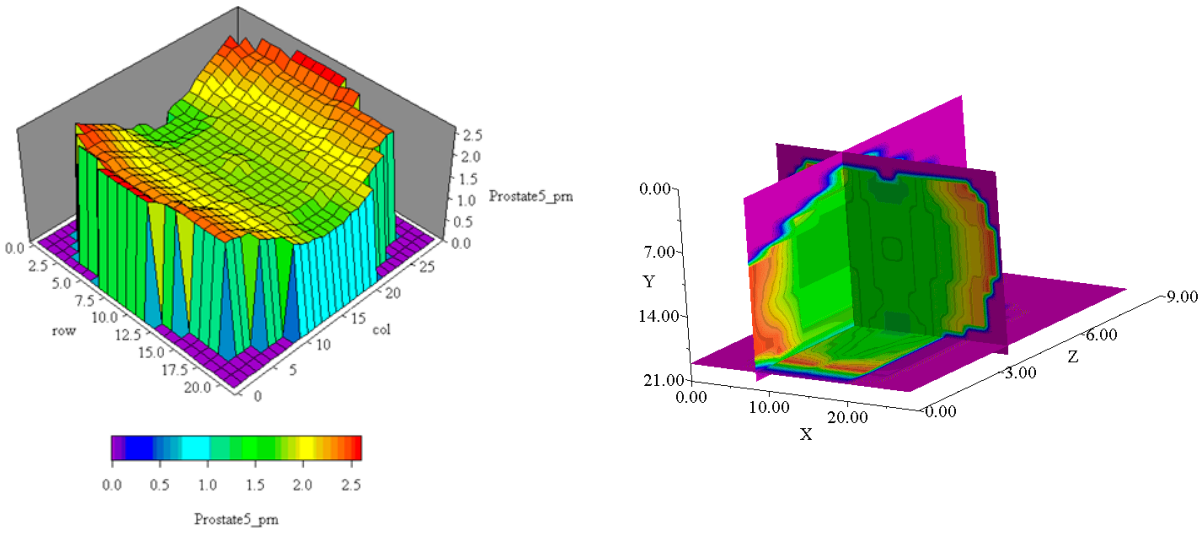

Figure 3. *Left picture*: Two-dimensional distribution of SnET2 within a canine prostate. *Right picture*: Three dimensional distribution of photosensitiser. Both images show the highest concentration of the drug in the peripheral regions of the gland [17]

Complete ablation will depend on precise placement of the light sources in the affected tissue, and the delivery of a therapeutic light dose. This will depend on a sequence of events: acquiring a three-dimensional tumour model, simulation to optimise the placement of the light sources, interstitial placement of the light sources, measurement of the above parameters of the treated organ, PDT computer simulation, evaluation of the treatment model and adjusting the parameters if needed, and finally executing PDT treatment based on all of these preparations.

PDT is not a simple treatment that can be done by eyeballing. It is a procedure that requires precise planning, which can be done with the help of complex computer programs [24–31]. Computer simulation of PDT to optimise treatment depends heavily on intense calculations in all steps of this procedure, and desktop computers are only now sufficiently powerful to assist physicians during therapy in real-time, to enable this therapy to treat a broad spectrum of malignancies.

Acknowledgements

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
There are no sources of funding to declare.

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Acceptance for editing: 2019-05-09
Acceptance for publication: 2019-11-28

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