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Neuroscience and Respiration

Mieczyslaw Pokorski *Editor*

Current Concepts in Medical Research and Practice

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Preface

The book series Neuroscience and Respiration presents contributions by expert researchers and clinicians in the multidisciplinary areas of medical research and clinical practice. Particular attention is focused on pulmonary disorders as the respiratory tract is up front at the first line of defense for organisms against pathogens and environmental or other sources of toxic or disease-causing effects. The articles provide timely overviews of contentious issues or recent advances in the diagnosis, classification, and treatment of the entire range of diseases and disorders, both acute and chronic. The texts are thought as a merger of basic and clinical research dealing with biomedicine at both the molecular and functional levels and with the interactive relationship between respiration and other neurobiological systems, such as cardiovascular function, immunogenicity, endocrinology and humoral regulation, and the mind-to-body connection. The authors focus on modern diagnostic techniques and leading-edge therapeutic concepts, methodologies, and innovative treatments in both adults and children. Practical, data-driven options to manage patients are considered.

Body functions, including lung ventilation and its regulation, are ultimately driven by the brain. However, neuropsychological aspects of disorders are still mostly a matter of conjecture. After decades of misunderstanding and neglect, emotions have been rediscovered as a powerful modifier or even the probable cause of various somatic disorders. Today, the link between stress and health is undeniable. Scientists accept a powerful psychological connection that can directly affect our quality of life and health span. Psychological approaches, which can decrease stress, can play a major role in disease therapy.

Disorders related to gene polymorphism and epigenesis, involving both heritable and non-heritable but functionally relevant changes in the nucleotide sequence of the genome are also tackled.

Clinical advances stemming from molecular and biochemical research are but possible if research findings are translated into diagnostic tools, therapeutic procedures, and education, effectively reaching physicians and patients. All this cannot be achieved without a multidisciplinary, collaborative, bench-to-bedside approach involving both researchers and clinicians. The role of science in shaping medical knowledge and transforming it into practical care is undeniable.

Concerning respiratory disorders, their societal and economic burden has been on the rise worldwide, leading to disabilities and shortening of life-span. Chronic obstructive pulmonary disease (COPD) alone causes more than three million deaths globally each year.

Concerted efforts are required to improve this situation, and part of those efforts are gaining insights into the underlying mechanisms of disease and staying abreast with the latest developments in diagnosis and treatment regimens. It is hoped that the articles published in this series will assume a leading position as a source of information on interdisciplinary medical research advancements, addressing the needs of medical professionals and allied health-care workers, and become a source of reference and inspiration for future research ideas.

I would like to express my deep gratitude to Paul Roos, Tanja Koppejan, and Cynthia Kroonen of Springer Nature NL for their genuine interest in making this scientific endeavor come through and in the expert management of the production of this novel book series.

Mieczyslaw Pokorski

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Electronic Cigarettes and Awareness of Their Health Effects

A. Daniluk, A. Gawlikowska-Sroka, M. Stępien-Słodkowska, E. Dzięciołowska-Baran, and K. Michnik

Abstract

The use of electronic cigarettes or e-cigarettes is strongly on the rise. The literature confirms that in the process of quitting smoking using an electronic device dispensing nicotine should be a transitional stage before the complete cessation of smoking. The aim of the present study was to assess the popularity of e-cigarettes, the underlying reasons for use of such nicotine products, and the level of awareness of health hazards associated with e-cigarettes. The study is of a survey type. The material consisted of data collected from an anonymous survey distributed among 46 female and 23 male users of e-cigarettes in 2015. We used a questionnaire of our own design. The findings demonstrate that the main reason for a recourse to e-cigarettes is a desire to use fashionable technological innovations, and the conviction that such cigarettes are less harmful than the traditional tobacco products. Some respondents used e-cigarettes to quit smoking; others to minimize the harmful effects of smoking. Most respondents acquired information about e-cigarettes from friends or from the Internet. There was a high awareness of the chemical composition of substances contained in e-cigarettes. An interest in e-cigarettes is caused by an increased knowledge on the negative effects of traditional smoking. Currently, the e-cigarettes remains a technological novelty, so that the exact health effects of their long-term use are open to conjecture.

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Keywords
Addiction • E-cigarettes • Health effect • Smoking cessation • Survey • Tobacco smoking

1 Introduction

The electronic cigarette or e-cigarette was invented by Hon Lik, a Chinese pharmacist, in 2003 (Kośmider et al. 2012). Within the last several years the interest of consumers in this product has appreciably increased. Currently, e-cigarettes are widely available worldwide. It has been estimated that over one billion people smoke traditional tobacco cigarettes, mainly in developing countries. The introduction of e-cigarettes on the global scale decreased the sale of classic tobacco cigarettes. In Poland, a steady, albeit slow, decline in the number of smokers has been observed since 1997, before the era of e-cigarettes. Currently, *ca* 29% of the entire population smoke cigarettes and the interest in e-cigarettes is sharply on the rise (Królikowski and Domagała-Kulawik 2014). In Europe and the US, it has been estimated that about 10 m people use this a nicotine delivery system. Initially, e-cigarettes were sold and advertised mainly *via* the Internet, but over time specialist shops offering these products have cropped up, particularly in popular shopping malls. The most recent studies have indicated that the majority of marketing activities rely on emphasizing the financial aspects of using e-cigarettes as a less expensive tobacco replacement product, compared with trade mark medications, and the health-related benefits of these devices, such as the lack of carcinogenic tar, and an aid in quitting smoking traditional cigarettes (Zarobkiewicz et al. 2016). A growing interest in e-cigarettes results from their ready availability and the lack of uniform legislation in this area, which varies from country to country (Sanders-Jackson et al. 2016). Initially, there was a belief that e-cigarettes are not tobacco products because they do not contain tobacco but just nicotine obtained from tobacco. Currently, criticism is expressed concerning that belief. In Australia, Brazil, and Finland the sale of e-cigarettes is prohibited. In Latvia, e-cigarettes may only be purchased by consumers aged 18 or older. In Hong Kong, regulations on e-cigarettes are the most restrictive because these products cannot be legally purchased or possessed. However, in New Zealand, Malaysia, or Austria e-cigarettes have a status of a medical device and are sold by prescription only. The UK and Germany have not established any specific regulations in this respect. In Poland, an act amending the law on the protection of public health against the effects of tobacco use was adopted on 22 July 2016. According to the currently existing law, e-cigarettes can only be purchased by people aged 18 or older. Akin to traditional tobacco products, the use of e-cigarettes in public places and the advertising of such cigarettes is prohibited. The Polish law has also introduced a ban on the on-line sales of most tobacco-related accessories, as well as some restrictions regarding conventional sales in shops.

The e-cigarette is a device that is used for the delivery of nicotine *via* the inhalation route. The device heats the e-liquid and transforms it into vapor. E-liquids used in e-cigarettes are either synthetic or natural. E-liquid usually contains polypropylene glycol, vegetable glycerine, flavor and its carriers, nicotine, preservatives, making up 95% of it, and some other additives accounting for the remaining 5%. Some e-liquids also contain colorants that do not interfere with the process of combustion. Most studies published to date have indicated a low toxicity of the substances above outlined (Golli et al. 2016). E-liquid does not contain carcinogens such as benzene or toluene, but recent studies underscore the probably presence of other highly harmful substances such as formaldehyde. Smokers

often look for e-cigarettes having a pleasant fragrance, which counters some unpleasant side effects of smoking tobacco cigarettes, such as smelly breath or stinky clothes. Users of e-cigarettes often report an improvement in smoking-related symptoms such as shortness of breath and cough, and point to having a better chance of fighting the habit of smoking traditional cigarettes (Królikowski and Domagała-Kulawik 2014). The e-cigarette is still a relative novelty on the health-related market, so that the long-term effects of using these devices are mostly unsettled and are subject to intense research. The present study seeks to define the level of e-cigarettes use in the general Polish population, the motivations behind the switch from smoking traditional cigarettes to e-cigarettes, and the level of awareness of e-cigarette smokers concerning the potential health risks. We addressed this issue across various age-groups of smokers in a survey-type study.

2 Methods

The study material consisted of data collected from an anonymous questionnaire-based diagnostic survey. The questionnaire was of our own design and it was designed according to the principles described in relevant publications (Babbie 2009). Data were collected from 69 respondents. The surveyed group comprised 46 women (67%) and 23 men (33%). The demographic characteristics of respondents are presented in Table 1. The questionnaire was completed either on-line (9 individuals) or in person by customers shopping for e-cigarettes at stalls specializing in selling them, usually present in shopping malls (60 individuals). Data were statistically elaborated with the help of a MS Excel spread sheet.

3 Results

Respondents participating in the survey were asked about their source of knowledge on e-cigarettes.

Table 1 Demographic characteristics of the study population

Age-group (year)	**Gender (Femal/Male; *n*)**
< 18	1/0
18–20	1/1
21–30	26/12
31–40	9/5
41–50	4/2
51–60	3/3
> 60	2/0
Education (n = 69)	**(*n*)**
Primary school	2
Vocational	4
High school	32
University	31

Most of them indicated friends (62%); others indicated the Internet (19%), television and press (6%). The majority of respondents (61%) stated that e-cigarettes were a better choice than tobacco cigarettes. Eighty-seven percent smoked tobacco cigarettes for 1 year up to 40 years before using e-cigarettes. Sixty-one percent of respondents had tried to quit smoking before using e-cigarettes. Sixty-one percent of respondents smoked tobacco cigarettes but at the same time they used innovative products with nicotine e-liquids. Most respondents found e-cigarettes as fashionable and less harmful to health (Fig. 1). The respondents differed concerning the awareness about health risks associated with the use of e-cigarettes. The opinion of the majority was that these devices were less harmful to health compared to tobacco cigarettes (Fig. 2).

Many of the respondents, however, despite their being regular users of the devices, were unfamiliar with the exact chemical composition of e-liquids they inhaled in the vaporized form. They used e-liquids containing from 6 to 18 mg of nicotine *per* ml of fluid, although there are e-liquids containing higher concentrations of nicotine available on the market. They used from one to six containers of e-liquid per month, each of 10 to 30 ml volume. Most of them declared the financial savings they made through owing to the use of e-cigarettes instead of tobacco cigarettes, ranging from about 15 to 100 euros *per* months. Respondents who completed questionnaires gave

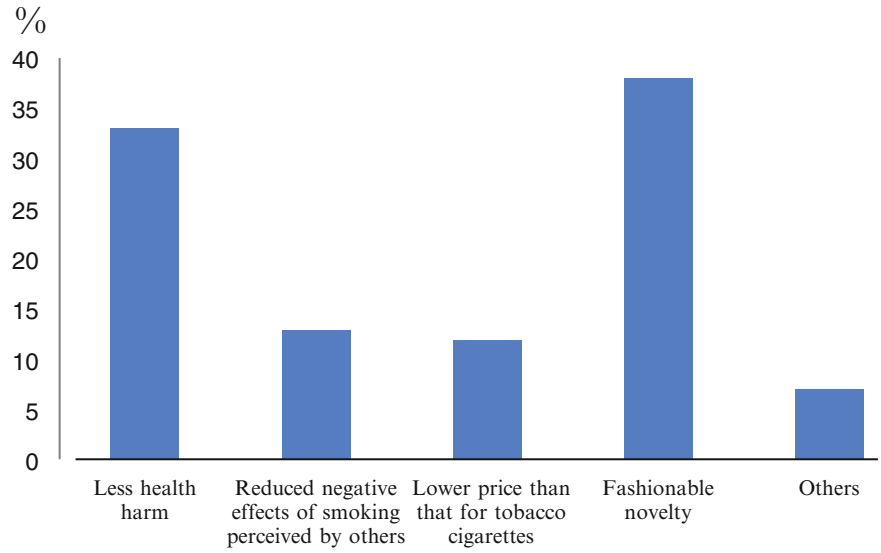
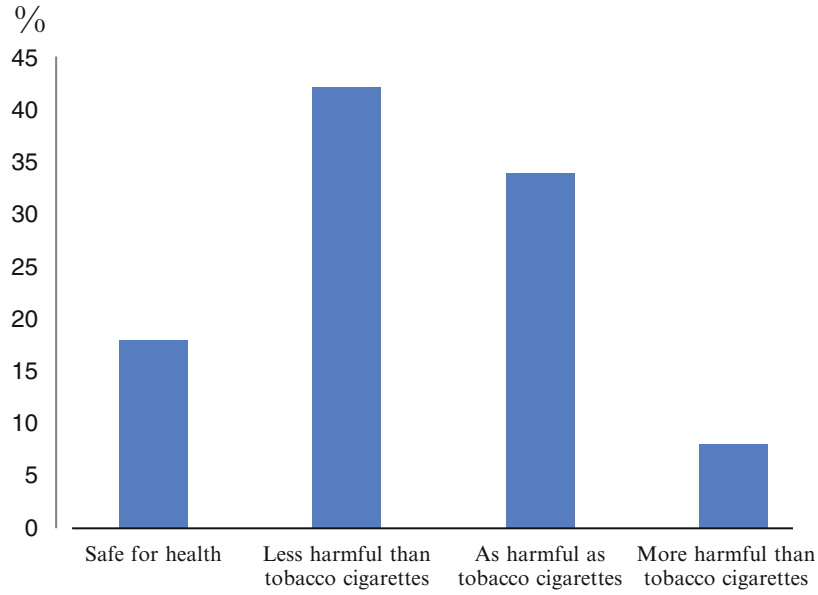


Fig. 1 Rationale for using e-cigarettes

Fig. 2 Knowledge of respondents on health risks associated with the use of e-cigarettes



various reasons for the ban of smoking e-cigarettes in public places. The majority of them indicated the harmfulness of e-cigarettes as being comparable to that of passive smoking of tobacco cigarettes. Psychological, esthetic, and cultural issues were rarely raised by the respondents as a reason to ban the e-cigarettes (Fig. 3). The majority of the respondent also conceded that they knew the reasons why e-cigarettes should be banned (Fig. 4).

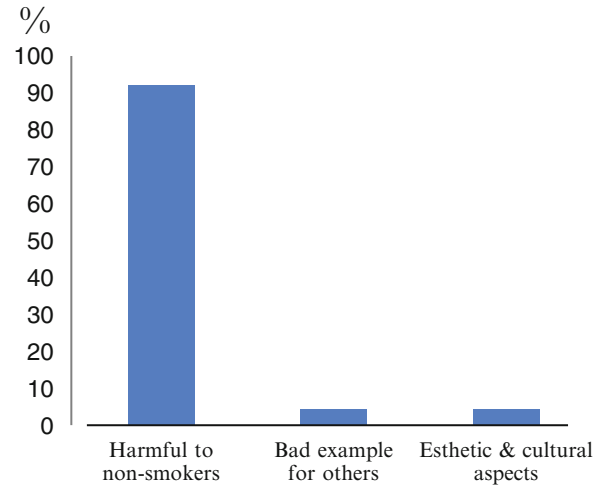


Fig. 3 Knowledge of respondents on the health and otherwise effects of chemical composition of e-liquids in e-cigarettes

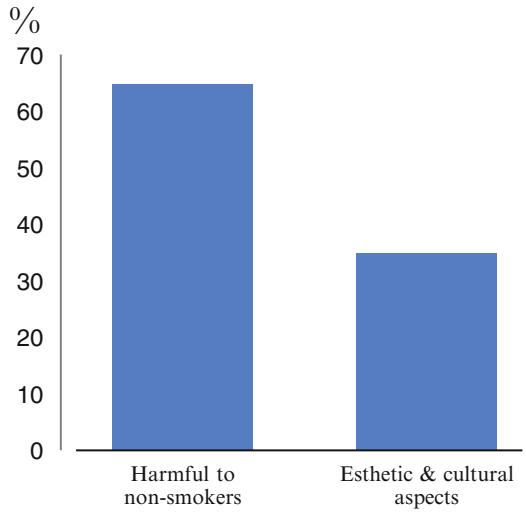


Fig. 4 Respondents' subjective knowledge of reasons for banning the use of e-cigarettes

4 Discussion

In recent years, a growing interest in e-cigarettes in both Poland and other countries has been observed (Zarobkiewicz et al. 2016; Rostron et al. 2016), which can be attributed to very effective marketing campaigns. E-cigarettes are advertised as a healthier option than tobacco cigarettes, cheaper, very easy to handle, and a very effective way to quit tobacco smoking (Aiche and Frishman 2016; Chatham-Stephens et al. 2016; Payne et al. 2016; Volesky et al. 2016). In Poland, until July 2016, when the law restricting the sales and use of e-cigarettes was introduced, these products had been praised as an alternative to tobacco cigarettes that can be legally used in public places. The strong interest is due also to a general increase in the social knowledge and awareness about the negative effects of tobacco smoking. Heavy smokers are looking for a way to quit smoking, as well as for replacement products regarded by society as less harmful. A reduction in symptoms such as coughing and shortness of breath after the switch from tobacco cigarettes to e-cigarettes causes an increasing interest in this form of nicotine delivery (Królikowski and Kulawik 2014). The use of e-cigarettes is also stimulated by current fashion. The literature still does not provide conclusive data explaining whether this electronic nicotine delivery system is a better, healthier alternative to tobacco cigarettes (WHO 2009). It is possible that future e-cigarettes will become a recognized aid in the fight against addiction. Smoking tobacco cigarettes, due to the presence of many harmful substances, leads to progressive physical damage manifested in pathological changes to organs and systems. Users of e-cigarettes do not inhale carbon monoxide that reduces the level of oxygen in the blood, tar, or other chemicals

released during tobacco combustion. They do inhale, however, other possibly toxic chemical substances contained in the e-liquid. Manufacturers of the e-liquids which are used in e-cigarettes claim that their products are effective in quitting tobacco smoking, and are much safer for health. Studies that have so far been conducted show large differences in reported results. For example, some researchers have found e-cigarettes to be safe products, but others show that e-cigarettes can be harmful to health and even carcinogenic because e-liquids, apart from nicotine, contain other chemicals that are heated to a temperature of 200 °C. For instance, vaporized e-liquid contains traces of carcinogenic formaldehyde and acrolein (Golli et al. 2016). Little is known about the chemicals contained in e-liquids. Certainly, the symptoms of nicotine dependence are also observed in users of e-cigarettes, although they are less pronounced than in tobacco smokers (Rostron et al. 2016).

Nicotine contained in e-liquids affects the sympathetic nervous system by increasing blood pressure, accelerating heart rate, and increasing oxygen consumption by the myocardium. This may also contribute to the narrowing of coronary arteries, and consequently reduce blood flow (Bandiera et al. 2016). Unfortunately, information about the toxicity of the vapor created in e-cigarettes is still inconclusive. The lack of standardized studies in this area makes it difficult, even impossible, to compare products from different manufacturers in terms of their effects on health (Orr 2014). Some manufacturers also offer nicotine-free e-liquids. Nevertheless, chemicals contained in these fluids penetrate to the human body (Holbrook 2016; Li et al. 2016). During pregnancy, smoking e-cigarettes is as dangerous as smoking tobacco, since fetal cells are very sensitive to any external factors (Chivers et al. 2016). It cannot be claimed with absolute certainty that e-cigarettes have a negative influence on the development of a baby in the mother's womb. However, a pregnant woman should be aware that everything that enters her body accumulates in the fetal body. The intake of nicotine can cause endocrine disorders in the child and can negatively affect the development of the circulatory system. It also leads to a rapid increase in blood glucose level, which can result in pancreatic disorders and the development of diabetes mellitus. A healthy body is usually able to cope with such fluctuations, unlike the body of the developing child (Chivers et al. 2016; Holbrook 2016). The currently observed level of dependence on e-cigarettes is much lower than that of tobacco cigarettes. This is attributed, among other things, to the different rates at which nicotine is released into the bloodstream. Nicotine from traditional cigarettes penetrates into the bloodstream in less than 5 min. The penetration time for nicotine from e-cigarettes is longer than that, but still shorter compared to chewing gum containing nicotine, which is about 25 min. Substances contained in the vapor produced by e-cigarettes include nicotine at varying concentrations, which is an addictive psychoactive substance. Therefore, e-cigarettes are also addictive, but it is widely believed that the level of this dependence is low (Kaisar et al. 2016; Sanders-Jackson et al. 2016).

The emergence of a new product replacing tobacco cigarettes has stimulated many different opinions regarding their positive or negative impact on the body of dependent users. Research carried out to date does not allow for a conclusive assessment of the effects caused by the long-term use of e-cigarettes. Until this year, such a situation was facilitated by the lack of legislative solutions limiting the access of underage persons to such nicotine delivery devices. The results obtained in the present study revealed a great popularity of e-cigarettes among residents of the city of Szczecin in Poland. Respondents participating in the survey acquired information about these devices mostly from friends, who themselves have probably used such products. Their knowledge also came from the Internet, television, and newspapers. The majority of respondents argued, in line with numerous but mostly non-scientific sources, that the e-cigarette it is a better choice than the tobacco cigarette. Respondents reached for these products after a period of addiction that ranged from 1 to 40 years. Most of them had made unsuccessful

attempts to quit smoking before they started using e-cigarettes. Many respondents continued to smoke tobacco cigarettes but also vaped nicotine. Some smokers indicated a current fad as a reason for inhaling the chemical substances contained in e-liquids. Respondents differed in terms of awareness about the health-related risks associated with the use of e-cigarettes. Most of them argued that these products are less harmful to health compared to tobacco cigarettes. Many people, despite the regular use of novel preparations, were unfamiliar with the chemical composition of the e-liquids they inhaled. A lower product purchase price is another reason given by many respondents in favor of using e-cigarettes. A study of Korzeniowska et al. (2014) has revealed that as many as 96% of respondents start using e-cigarettes due to their lower cost compared to tobacco cigarettes. A very alarming fact has been reported by Bold et al. (2016), namely that young people trying e-cigarettes are strongly predisposed to become heavy smokers. It is also disturbing that the age of e-cigarette users is decreasing (Hammig et al. 2016). That points to the need of developing prevention campaigns and legislation aimed at limiting this negative trend in the population (Bold et al. 2016; Richter et al. 2016). Poland has introduced the law on the protection of public health against the effects of tobacco use in July of 2016, banning smoking tobacco, including innovative tobacco products and e-cigarettes in public places. The law also enforces placing information on the harmful effects of substances contained in e-cigarettes on the human body, and prohibits selling these devices to persons under age 18. The insufficient knowledge of the health effects of e-cigarettes is stimulating further research in this area. It seems necessary to analyze in detail the composition of e-liquids available to consumers and each new e-liquid before its launch on the market.

In conclusion, the e-cigarette is a technological novelty, so that the long-term health effects of e-cigarette smoking remain as yet unsettled. The ban on smoking tobacco cigarettes in public places changes the behavior of smokers and makes them search for other nicotine delivery systems to satisfy the needs of a nicotine-dependent body.

Conflicts of Interest The authors declare no conflict of interest in relation to this article.

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Metachronous Lung Cancer: Clinical Characteristics and Effects of Surgical Treatment

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Abstract

The occurrence of a second lung tumor after surgical removal of lung cancer usually indicates a lung cancer metastasis, but sometimes a new lesion proves to be a new primary lung cancer, i.e., metachronous lung cancer. The goal of the present study was to conduct a clinical evaluation of patients with metachronous lung cancer and lung cancer metastasis, and to compare the early and distant outcomes of surgical treatment in both cancer types. There were 26 age-matched patients with lung cancer metastases and 23 patients with metachronous lung cancers, who underwent a second lung cancer resection. We evaluated the histological type of a resected cancer, the extent of thoracosurgery, the frequency of early postoperative complications, and the probability of 5-year survival after the second operation. The findings were that metachronous lung cancer was adenocarcinoma in 52% of patients, with a different histopathological pattern from that of the primary lung cancer in 74% of patients. In both cancer groups, mechanical resections were the most common surgery type (76% of all cases), with anatomical resections such as segmentectomy, lobectomy, or pneumectomy being much rarer

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conducted. The incidence of early postoperative complications in metachronous lung cancer and lung cancer metastasis (30% *vs.* 31%, respectively) and the probability of 5-year survival after resection of either cancer tumor (60.7% *vs.* 50.9%, respectively) were comparable. In conclusion, patients undergoing primary lung cancer surgery require a long-term follow-up due to the risk of metastatic or metachronous lung cancer. The likelihood of metachronous lung cancer and pulmonary lung cancer metastases, the incidence of postoperative complications, and the probability of 5-year survival after resection of metachronous lung cancer or lung cancer metastasis are similar.

Keywords

Histopathology • Lung cancer • Metachronous cancer • Metastasis • Non-small cell lung cancer • Survival • Thoracic surgery • Tumor

1 Introduction

The appearance of another neoplastic focus after resection of non-small cell lung cancer (NSCLC) is an important clinical problem and requires a differentiation between lung cancer metastases and a second primary lung cancer, i.e., metachronous lung cancer. The classic diagnostic criteria, based on clinical and histological data, enabling the distinguishing between the lung cancer metastasis and metachronous lung cancer have been established by Martini and Melamed (1975) and are so far used in practice. Metachronous lung cancer most often has a different histological structure than primary lung cancer or, in case of a similar structure, metachronous lung cancer diagnostic criteria include the occurrence at least 2 years following primary lung cancer, *in situ* development, localization in another lobe, lack of the same path of spreading, i.e., no tumor lesions in lymph nodes, and no other extrapulmonary metastases.

Surgical treatment differs in scope in lung cancer metastasis and metachronous lung cancer. In case of primary or metachronous lung cancer, anatomic resection with removal of three layers of lymph nodes of the pulmonary hilum and mediastinum is necessary (Gamliel 2016; Maniwa and Kodama 2016; Riquet et al. 2016; Hytych et al. 2013; Asamura et al. 1999; Riquet et al. 1994; Naruke et al. 1988). On the other hand, wedge resection is most often employed in case of lung cancer metastasis, followed only by sampling of potentially affected lymph nodes (Sihag and Muniappan 2016). In general, resection of a second pulmonary neoplasm, irrespective of its histopathological origin, presents an enhanced risk of postoperative complications and may worsen the long-term outcome.

The goal of the present study was to conduct a clinical evaluation of patients with metachronous lung cancer and with lung cancer metastasis, and to compare the early and distant outcomes of surgical treatment in both lung cancer entities.

2 Methods

The study was approved by the Ethics Committee of Wroclaw Medical University in Poland and it was conducted in accord with the principles of the Declaration of Helsinki for Human Research of the World Medical Association. The research material for the study consisted of 49 thoracosurgical patients, a random sample chosen from 6162 patients suffering from primary lung cancer metastasis or a second metachronous lung cancer, operated on in the years 2001–2015 in the Lower Silesian Center of Lung Diseases in the city of Wroclaw, Poland. There were 26 patients (53%) with lung cancer

metastases and 23 patients (47%) with metachronous lung cancers. Gender and age of patients, and the stage of lung cancer were similar in both groups. Adenocarcinoma was the most common cancer type in both groups. A few more patients with lung cancer metastasis had more than one metastasis. The detailed figures are given in Table 1.

Diagnostic tests performed in all patients prior to surgery included the following: bronchofiberoscopy, chest X-ray, thoracic computed tomography, and abdominal ultrasonography. Since 2007, also positron emission tomography (PET) was performed. Mediastinoscopy was performed in case of the possible involvement of mediastinal lymph nodes, i.e., their enlargement of more than 10 mm or grouping into packages seen in the imaging scans. Since 2008, mediastinoscopy was replaced with the needle biopsy of mediastinal nodes under the endobronchial ultrasonography (EBUS) control. Surgical treatment was abandoned in case of mediastinal lymph node or remote metastases.

The surgical procedures employed are listed in Table 2. In both groups, mechanical wedge, tangent, and laser resections were the most common – in 76% of all cases. Anatomical resections consisting of segmentectomy or lobectomy were rarer – in 16% of all cases, and pneumectomy was conducted only in individual cases. The examples are illustrated in Figs. 1 and 2. The resection failed to be radical in two patients treated for lung cancer metastasis. Lymphadenectomy was performed with similar frequency in both groups of patients; in 43% cases in total (Table 2).

Continuous data were presented as means ±SD or medians, as indicated, and discrete data as counts and percentages. The Mann-Whitney U was used to assess differences between the two

Table 1 Demographics, histological diagnosis, stage, and the number of primary tumors in patients operated on due to primary lung cancer metastasis (LCM) and metachronous lung cancer (MLC)

	LCM (n = 26)	MLC (n = 23)[a]
Age (year)	66.4 ± 4.9	66.5 ± 5.1
Range (year)	50–77	54–79
Men	16 (62%)	15 (65%)
Women	10 (38%)	8 (35%)
Adenocarcinoma	15 (58%)	12 (52%)
Squamous cell carcinoma	9 (35%)	6 (26%)
Other histological types	2 (8%)	5 (22%)
Stage I	16 (62%)	17 (74%)
Stage II	4 (15%)	3 (13%)
Stage III	3 (11.5%)	2 (9%)
Missing data on staging	*3 (11.5%)*	*1 (4%)*
T1	12	14
T2	10	8
T3	1	0
T4	1	1
Tx	2	0
N0	19	18
N1	2	2
N2	2	2
Missing data on N	*3*	*3*
1 tumor	20 (77%)	20 (87%)
≥ 2 tumors	6 (23%)	2 (9%)
Missing data on number of tumors	*0*	*1*

Cancer staging was performed according to Edge and Compton (2010)

[a]All differences between MLC and LCM patients failed the test of significance at $p < 0.05$

Table 2 Surgical treatment in patients with primary lung cancer metastases (LCM) and metachronous lung cancer (MLC)

	LCM (n = 26)	MLC (n = 23)	*p*
Surgery type			
Mechanical resection	18 (69%)[a]	19 (83%)	ns
Segmentectomy	3 (11%)	1 (4%)	ns
Lobectomy	2 (8%)	2 (9%)	ns
Pneumectomy	1 (4%)	1 (4%)	ns
Non-radical resection	2 (8%)	0 (0 %)	ns
Surgery side			
Right-sided surgery	10 (38%)	16 (70%)[b]	0.03
Left-sided surgery	16 (62%)	7 (30%)[b]	0.03
Lymph node N1 or N2 surgery			
Resected	10 (38%)	11 (48%)	ns
Non-resected	16 (62%)	12 (52%)	ns

[a]including one mechanical resection combined with radical segmental resection of a rib
[b]significant difference between LCM and MCL groups, *ns*, non-significant difference

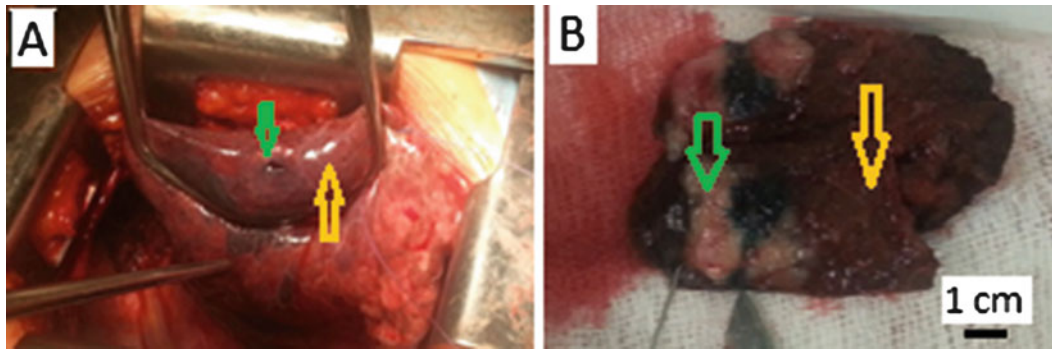

Fig. 1 (**a**) Wedge resection of tumor (*green arrow*) with a margin of lung parenchyma (*yellow arrow*). In the resection area there usually are the lymph nodes of groups 14, 13, and sometimes 12; (**b**) resected tumor tissue (*green arrow*), with a margin of lung parenchyma (*yellow arrow*)

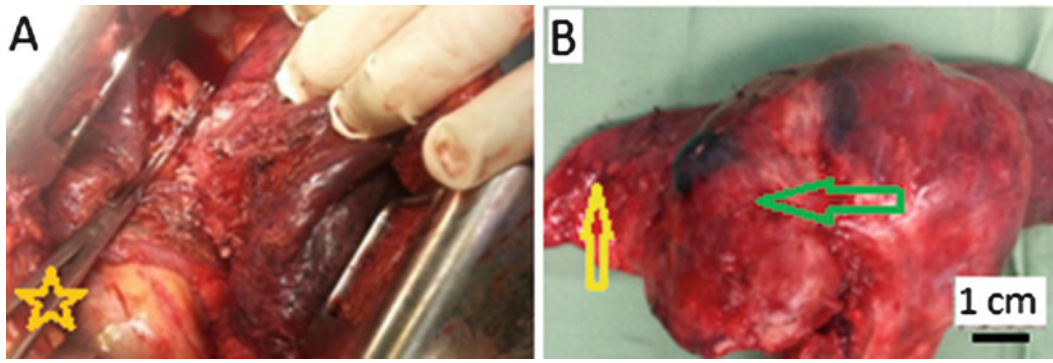

Fig. 2 (**a**) Anatomical resection: postoperative loge with Satynski clamp (*yellow star*) closing the bronchus stump is seen in the left-hand part of the photograph. Anatomical resection usually involves removal of the lymph nodes of groups 11, 10, and the mediastinal nodes of group 7 (**b**) Resected lung lobe with cancer foci (*green arrow*); atelectatic neighboring lung parenchyma (*yellow arrow*)

independent groups of patients and the Chi-squared test to compare features between the groups such as histopathological changes, cancer stages, and surgical treatments. The Kaplan-Meier estimator was used to assess the probability of patient survival, and the difference between the two survival curves was assessed with the Mantel-Cox test. A Cox regression analysis also was performed to determine the difference in patient survival with respect to clinical and pathological data. A p-value < 0.05 defined the statistically significant differences. Commercial StatSoft v1.3 (Statsoft, Cracow, Poland) and GraphPad Prism v5.0 (La Jola, CA) statistical packages were used for all data analysis.

3 Results

Among 49 patients who underwent the second resection of a lung cancer, metachronous cancer was diagnosed in 23 (47%) of patients. A histopathological examination revealed a different cellular organization of cancer tissue, compared with primary lung cancer, in 17 patients (77%). In the remaining six patients metachronous cancer was histologically the same as the primary tumor. However, since the metachronous cancer appeared after more than 2 years from the detection and surgery of the primary tumor, it was considered metachronous. The detailed data are presented in Table 3.

The median time elapsing from the resection of a primary tumor to lung cancer metastasis resection was 24.5 months and it was significantly shorter than that elapsing from the resection of a primary tumor to metachronous lung cancer resection, which was 49 months ($p < 0.05$). The early results of surgical treatment in patients treated for both lung cancer metastasis and metachronous lung cancer were similar (Table 4). The incidence of postoperative complications was noted in 31% patients with lung cancer metastasis and 30% patients with metachronous lung cancer.

Table 3 Histological type of metachronous lung cancer and primary lung cancer in the same patient (n = 23)

Metachronous cancer	Primary cancer	n
Squamous cell carcinoma[a]	Squamous cell carcinoma	**2**
Mixed adenocarcinoma and squamous cell carcinoma	Squamous cell carcinoma	2
Adenocarcinoma	Squamous cell carcinoma	4
Unspecified	Squamous cell carcinoma	1
Large cell carcinoma	Squamous cell carcinoma	1
Squamous cell carcinoma	Adenocarcinoma	3
Large cell carcinoma	Adenocarcinoma	1
Adenocarcinoma[a]	Adenocarcinoma	**4**
Adenocarcinoma	Neuroendocrine carcinoid	1
Adenocarcinoma	Large cell carcinoma	1
Adenocarcinoma	Unspecified	2
Squamous cell carcinoma	Unspecified	1

[a]New tumor unraveled after more than 2 years from the previous cancerous episode; although histologically same, was considered metachronous cancer; figures in bold depict these six histologically same cases

Table 4 Early postoperative complications in patients with primary lung cancer metastases (LCM) and metachronous lung cancer (MLC)

Complication	LCM (n = 26)	MLC (n = 23)
Atelectasis caused by bronchial secretion	0	1 (4%)
Unexpandable lung	6 (23%)	3 (13%)
Cardiac arrhythmias and circulatory insufficiency	1 (4%)	2 (9%)
Bleeding into the post-treatment chamber	1 (4%)	1 (4%)

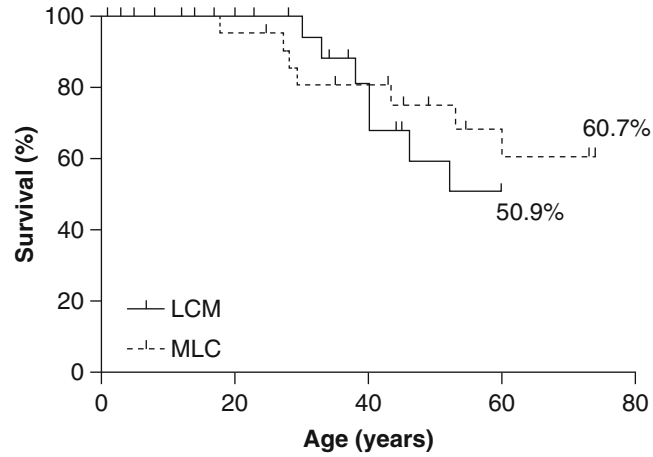

Fig. 3 Probability of 5-year survival in patients with primary lung cancer metastases (LCM) and with metachronous lung cancer (MLC)

Table 5 Prognostic factors in patients with metachronous lung cancer (MLC) – univariate Cox regression analysis.

Risk factor	RR (95%CI)	*p*
Size of metachronous lung cancer	0.99 (0.93–1.06)	0.81
Localization: Intrapulmonary or subpleural	0.80 (0.19–3.35)	0.76
Co-morbidities	1.00 (0.98–1.01)	0.67
Age of patient	0.93 (0.82–1.05)	0.26

RR (95%CI) relative risk with the lower and upper limits of 95% confidence interval

The analysis of long-term surgery results showed that the probability of the 5-year survival rate in patients with lung cancer metastasis (50.9%) and those with metachronous lung cancer (60.7%) was similar. The survival results are displayed in Fig. 3. Concerning the prognostic factors in patients with metachronous lung cancer after surgical treatment we failed to demonstrate any effect of such factors as tumor size or its localization in the lung, age of patient, or co-morbidities on the survival rate (Table 5).

4 Discussion

The major finding of this study was that a second operation of lung cancer concerned metachronous lung cancer in 47% of cases, i.e., in about one half of operations occurring after surgical resection of the primary lung cancer; the other half being due to lung metastases of the primary cancer. Metachronous lung cancer was observed mostly in men and appeared, on average, 49 months after the first surgery. The majority of metachronous cancers were adenocarcinomas, and their histological pattern usually was different from that present in the primary lung cancer. Metachronous lung cancer was subject to mechanical resection in most cases. The outcome of surgical treatment of metachronous lung cancers was akin to that of lung cancer metastases, with a similar rate of complication (30% and 31%, respectively) and the similar probability of the 5-year survival rate (60.7% and 50.9%, respectively).

Martini and Melamed (1975) criteria were adopted in the present study for distinguishing between metachronous lung cancer and lung cancer metastasis, including the time lapse of at least 2 years between the resection of a primary lung cancer and the appearance of metachronous lung cancer. These criteria are commonly used, although they are sometimes subject to critical evaluation and modification. For example, some studies have adopted the criterion of at least a 4-year disease-free time after primary lung

cancer resection, which enables the diagnosis of metachronous lung cancer (Ha et al. 2015). In the present study, the mean time from resection of primary lung cancer to resection of metachronous lung cancer amounted to 4.6 ±2.1 years. Currently, the classical criteria for the diagnosis of metachronous lung cancer provided by Martini and Melamed (1975) are more often replaced by an extended imaging, histological, genetic, and molecular diagnostics (Stiles 2017; Liu et al. 2016). The differentiation of metachronous lung cancer from lung cancer metastasis, when both have the same histopathological cancer tissue structure, can be assisted with comparative genomic hybridization and somatic mutation testing (Arai et al. 2012; Girard et al. 2010; Moffat-Bruce et al. 2010; Wang et al. 2009). Genetic studies, however, have a limited value due to the possibility of different mutations in multiple tumors in the same patient. Such tests also are seldom employed since they are not commonly available and pricey.

The risk of metachronous lung cancer development in patients after NSCLC resection is 1–2% *per* patient *per* year (Johnson 1998; Johnson et al. 1997). The literature demonstrates that the incidence of metachronous lung cancer among patients operated on due to primary lung cancer is about 5% (Ishigaki et al. 2013; Vansteenkiste et al. 2013). In Poland, the incidence of multiple cancers, most commonly a second lung cancer, has also been reported at 5% in patients with lung cancer (Romaszko et al. 2016). In the present study, however, this risk appeared at just 0.4%, which may have been due to erratic and insufficient patient attendance to follow-up examinations after the surgery.

In our opinion, greater attention should be paid to the results of a histopathological examination of metachronous lung cancer. In the present study, adenocarcinoma was the most common histological metachronous cancer type, found in 57% of patients. Similar data on the adenocarcinoma prevalence among metachronous lung cancers are provided by other authors (Yang et al. 2014; Hamaji et al. 2013; Zuin et al. 2013).

The recommended method of surgical treatment of metachronous lung cancer is an anatomical resection with removal of regional lymph nodes (Wen et al. 2016; Zuin et al. 2013). In the present study, lymph nodes were removed in 48% of metachronous lung cancer cases. A low percentage of lymphadenectomy was often caused by a misleading treatment of metachronous lung cancer as lung cancer metastases. The decision on the extent of resection was made on the basis of an *ad-hoc* intraoperative inspection of a resected tumor; the inspection that usually is capable of providing only the information on the tumor's neoplastic character. The anatomical resection was performed in just 17% of cases metachronous lung cancer. In the present study, no patient passed away in the perioperative period. In literature, perioperative mortality associated with metachronous lung cancer resection ranges from 1.4% (Yang et al. 2014) to 2.5% (Zuin et al. 2013). We found other postoperative complications following metachronous lung cancer surgery in about one third of patients, as described also by other authors who noted the perioperative occurrence of complications ranging from 19% (Zuin et al. 2013) to 34.3% (Yang et al. 2014).

The probability of 5-year survival in the patients of the present study treated for metachronous lung cancer was evaluated as 60.7%. Almost the identical 5-year survival rate of 60.8% has been shown in a study of Hamaji et al. (2013). A higher survival rate of 69.5% has been shown in a most recent study of Zhao et al. (2017). In that study, however, only were the patients examined in whom metachronous lung cancer was of adenocarcinoma type. In other studies, the 5-year survival rate after surgical treatment of metachronous lung cancer has been calculated at a somehow lower level. Yang et al. (2014) have demonstrated a 54.5% survival rate, whereas Koezuka et al. (2015) have found it at 56.5%. Zuin et al. (2013) have demonstrated a 42% survival rate in 121 patients with metachronous lung cancer diagnosed according to Martini and Melamed's (1975) criteria. The 2014 meta-analysis of nine studies demonstrates

that the 5-year survival rate after surgery of a second primary NSCLC is 46% (Hamaji et al. 2015). There is a clear relationship between the 5-year survival rate and the stage of metachronous lung cancer (Koezuka et al. 2015), or the extent of surgery: from 57% in patients with lobectomy to 36% in patients who undergo segmentectomy or wedge resection (Zuin et al 2013). One of the prognostically adverse factors seems the size of metachronous lung cancer being resected (Hamaji et al. 2013). In the present study, however, tumor size was not a predicting factor for the 5-year survival rate.

In the present study, the 5-year survival rate after surgery for metachronous lung cancer was inappreciably greater than that for lung cancer metastases. In contrast, the 2015 meta-analysis that included 1,796 patients in 22 studies has demonstrated that the overall survival of patients with multiple primary lung cancers, both metachronous and synchronous tumors were taken into consideration, is longer than that in patients operated on due to intra-pulmonary lung cancer metastases; relative risk of 2.66 with 95% CI of 1.30–5.44, $p < 0.01$ (Jiang et al. 2015).

5 Conclusions

Among lung tumors arising after resection of the primary lung cancer, the likelihood of metachronous lung cancer is akin to that of pulmonary lung cancer metastasis. Surgically resected metachronous lung cancer is in most cases of adenocarcinoma type, and the histopathological pattern usually differs from that of the primary lung cancer. Patients who undergo primary lung cancer surgery require a long-term follow-up due to the risk of lung cancer metastasis or metachronous lung cancer. The incidence of early postoperative complications and the probability of 5-year survival after metachronous lung cancer and lung cancer metastasis resection are similar.

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Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Healthcare Professionals' Knowledge of Influenza and Influenza Vaccination: Results of a National Survey in Poland

Ernest Kuchar, Kamila Ludwikowska, Adam Antczak, and Aneta Nitsch-Osuch

Abstract

In Poland, the seasonal influenza vaccination rate is just barely 3% which may be related to the unsatisfactory knowledge of influenza among healthcare professionals, poor recognition of the benefits of influenza immunization and the fear of side effects. To address these issues, we surveyed healthcare professionals through an online questionnaire consisting of 18 closed-ended items. The questionnaire was completed by 495 healthcare professionals, mostly physicians (83%). The results revealed gaps in the knowledge concerning influenza diagnosis, complications, risk groups, and prognostic factors. On average, respondents only answered 4.8 of the 18 questions correctly (27%). Only 10% of respondents passed the threshold of 50% correct answers. The knowledge of contraindications to vaccination far outweighed the knowledge of indications for vaccination. Poor knowledge with a focus on the adverse effects of immunization may be a significant factor responsible for the low vaccination rate in Poland. To increase vaccination rate, healthcare professionals need to be educated about influenza-related risks and benefits of vaccination.

Keywords

Decision making • Healthcare professionals • Immunization • Influenza • Recommendations • vaccination

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1 Introduction

Influenza viruses are *Orthomyxoviridae* and are classified into three distinct types: A, B, and C. Epidemics of influenza A and B occur annually during the winter season in temperate regions of the northern hemisphere, including Poland, while influenza C viruses cause sporadic respiratory tract infections. Influenza viruses cause a broad spectrum of respiratory tract diseases, ranging from asymptomatic infection to pneumonia and acute respiratory distress syndrome, and they are responsible for significant morbidity, hospitalizations, and mortality worldwide. During the latest influenza season of 2016/2017, approximately 3.79 m cases of influenza-like illness were registered in Poland, with 13,000 hospitalizations and 24 deaths being attributed to influenza (National Influenza Center 2017).

Influenza A has a potential to cause global pandemics. Five pandemics occurred in the last century: A/H1N1 ('Spanish flu' in 1918), A/H2N2 ('Asian flu' in 1957), A/H3N2 ('Hong Kong flu' in 1968), A/H1N1 ('Russian flu' in 1977), and most recently, A/H1N1pdm09 ('Swine flu' in 2009) (RCPCH 2016; CDC 2015, 2016). The threat of a new influenza pandemic is always present. For the aforementioned reasons, knowledge of influenza, possible complications, treatment, and prevention is crucial for healthcare practitioners. The effective prevention with immunization and a rapid diagnosis, followed by administration of antivirals when necessary, and the isolation of infectious patients are fundamental for the limiting of influenza spread and burden. The annual influenza vaccination is the most effective preventive measure. Polish and other national guidelines are updated regularly and, in recent years, indications for the vaccine use have become broader and cover, apart from the healthy population aged over 6 months, such risk groups as pregnant women and immunocompromised individuals (Grohskopf et al. 2016). Despite the broad indications, influenza vaccination rate remains very low in Poland, amounting to 2.2–3.4% of the general population. In more detail, vaccination rate is about 9% among healthcare professionals, 0.5–1% in children aged 6 months to 4 years, and 7–13% among the elderly aged over 65 (Czarkowski et al. 2016). Since healthcare professionals are crucial to the implementation and execution of recommendations for the vaccination, its low coverage rate may be related to their unsatisfactory knowledge of influenza, poor recognition of immunization benefits, and unjustified fears of side effects. To address these issues, we examined physicians' knowledge of influenza, its complications and treatment, and the indications and contraindications to vaccination.

2 Methods

This survey-type study was approved by the Ethics Committee of Warsaw Medical University in Poland and it was conducted in accord with the principles of the Declaration of Helsinki for Human Research of the World Medical Association. The population sample surveyed consisted of 495 random healthcare professionals from Poland, mostly women (70%). Four hundred and eleven respondents (83%) were physicians, notably general practitioners, while rheumatologists and cardiologists were the most commonly represented subspecialists. Table 1 summarizes the basic demographic information and characteristics of the participants.

An online questionnaire consisting of 18 mostly multiple-answer, closed-ended items was designed explicitly for the purpose of this

Table 1 Demographics and professional qualifications of study participants

	n (%)
Gender	
Men	149 (30.1)
Women	346 (69.9)
Profession	
Doctors	411 (83.0)
Nurses	18 (3.7)
Medical students	20 (4.0)
Others	46 (9.3)

study by two members of the Polish Expert Committee of the National Program for Influenza Prevention. The items contained a variable list of correct choices. When more than one choice was correct, all correct choices in an item had to be checked off to include the item into correct responses. The questionnaire items, along with the responses provided by the interviewees, are displayed in Table 2. The survey was conducted on-line *via* social media or email among a varied group of healthcare professionals. The questionnaire was anonymous and voluntary, and the participants were informed about its aim. Answers were scored as correct based on published literature and current recommendations of the Advisory Committee on Immunization Practices (ACIP) of the Centers for Disease Control and Prevention (CDC).

3 Results

On average, respondents gave correct answers to 4.8 out of the 18 survey items, i.e., each item was correctly addressed by about a quarter of respondents (133/495 or 26.9%). The majority of correct responses (88.5%) concerned the contraindications to influenza vaccination and the recommendations for use of antivirals in influenza treatment (63.0%). More than half of respondents (56.6%) knew the cardiovascular indications for immunization against influenza, but a sunstantial number (44.4%) failed to recognize the general recommendations for immunization as set out in the Polish Immunization Program of 2014.

The knowledge of subspecialists about influenza virus was far from being sufficient as well, with just 5% of respondents being able to correctly name the virus type that was responsible for 'avian flu'. Only did 8.9% of respondents give correct answers concerning the transmission routes of influenza virus. Outstandingly, the majority appeared unaware of the possibility of vertical transmission, for instance from mother to child. The gaps in practical knowledge were particularly worrisome in some specific areas such as influenza complications in pregnant women (10.3% of correct answers), vaccine administration routes (11.3% of correct answers), influenza diagnosis tests (12.1% of correct answers), and interpretation of rapid test (13.9% of correct answers). Only did 9.7% of respondents give correct answers concerning the symptoms raising a specter of a severe or progressive course of influenza. Healthcare professionals also had a difficulty in defining the proper vaccine dosing in the pediatric population (16.3% of correct answers). However, a low number of pediatricians participating in the study (4% of respondents) could bear on this matter. Finally, only did 10% of respondents pass the survey with the threshold set at 50% of correct answers (Table 2).

4 Discussion

Every year, approximately 5–20% of the population acquires influenza. Although the majority of influenza infections are benign, self-limiting, and require only symptomatic care, a substantial number of cases result in complications, hospitalizations, and deaths. The analysis of long-term data in the US population of approximately 300 m people has revealed that the number of annual influenza-related deaths from respiratory and circulatory causes ranged from 3349 to 48,614, with an average of 23,607 deaths (CDC 2010, 2015). In the EU, the annual number of individuals of all ages infected with influenza is estimated at 25–100 m, with approximately 38,500 deaths (CDC 2016). In Poland, there were 3,793,770 cases of influenza and influenza-like illness reported in the most recent 2016/17 epidemic season, with 13,779 hospitalizations or 0.36% of patients being hospitalized, and 24 deaths. The incidence was estimated at 9842 *per* 100,000 people (National Influenza Center 2017). Although these figures change in a variable manner every next year, there is a consistent impression that the disease incidence increases (Table 3), which may likely be due to persistently low vaccination rate. These data, even though they are likely underestimated due to the imperfect, passive reporting system,

Table 2 Survey results

Items	(Multiple choice questionnaire; correct answers are underlined)	Correct answers *n* (%)	Incorrect answers *n* (%)
1	Indicate the most common complications of influenza in children:	120 (24.2)	375 (75.8)
	Otitis media		
	Myocarditis		
	Febrile seizures		
	Myositis		
2	Cardiovascular indications for immunization against influenza are:	280 (56.6)	215 (43.4)
	Isolated hypertension		
	Heart infarction in past		
	Ischemic heart disease		
	Heart failure		
3	Use of which of the following is a contraindication for influenza vaccination:	438 (88.5)	57 (12.5)
	Immunostimulants		
	Antiretroviral drugs		
	Topical corticosteroids		
	HIV infection		
	Renal transplantation performed 2 years earlier		
	None of the above		
4	Choose correct statements on isolation of influenza patients and influenza infectiousness according to CDC and WHO guidelines:	111 (22.4)	384 (87.6)
	5-days isolation in adults and 7-day isolation in children		
	7-day isolation is indicated for all persons with influenza or, if symptoms persist, until 24 h after their resolution		
	Viral shedding and duration of viral transmission is longer in children than in adults		
	Shedding the virus and infectiousness takes twice as long in adults than in children		
5	Choose correct statements about influenza vaccination in pregnant women:	140 (28.3)	355 (71.7)
	Vaccination is recommended for pregnant women in the second and third trimester		
	Vaccination is recommended regardless of trimester		
	Vaccination is not recommended for pregnant women		
	Frequency of vaccine's adverse effects is slightly higher in pregnant women than in the general population		
	Frequency of the vaccine's adverse effects is the same in pregnant women as in the general population		
6	Possible routes of influenza virus transmission are:	44 (8.9)	451 (91.1)
	Airborne – via droplets		
	Person-to-person contact		
	Vertical		
	Food borne		
7	Indicate the highly pathogenic avian influenza viruses	23 (4.7)	472 (95.3)
	A H7N9		
	A H5N1		
	A H7N7		
	A H1N1		

(continued)

Table 2 (continued)

Items	(Multiple choice questionnaire; correct answers are underlined)	Correct answers *n* (%)	Incorrect answers *n* (%)
8	According the polish immunization program, the indication for immunization covers which of the following groups:	220 (44.4)	275 (55.6)
	Pregnant women or those who intend to become pregnant		
	Chronically ill children (over 6 months of life) and adults; particularly those with respiratory insufficiency, asthma, chronic obstructive pulmonary disease, heart failure, ischemic heart disease, renal failure, nephritic syndrome, liver diseases, metabolic disorders including diabetes, neurological diseases and disorders (individual and clinical indications)		
	Immunosuppressed individuals (individual and clinical indication)		
	All persons over 6 months of life; particularly healthy children aged 6 months to 18 years of life (epidemiological indications)		
9	Indicate the relationship between influenza, vaccination and Guillain Barré syndrome (GBS)	89 (18.0)	406 (82.0)
	GBS occurring 6 weeks following vaccination is a contraindication to future vaccination		
	Risk of GBS is much lower after vaccination than after influenza infection		
	Risk of GBS is much higher after vaccination than after influenza infection		
	Risk of GBS after vaccination is akin to that after influenza infection		
	All the statements above are false		
10	Indicate the correct mode of influenza vaccine administration in a 5-year-old child who received the first dose of vaccine during the previous season:	81 (16.4)	414 (83.6)
	One 0.5 ml dose of influenza vaccine should be administrated during the current season		
	Two doses 0.25 ml of influenza vaccines 4 weeks apart, should be administered during the current season		
	Two doses 0.5 ml of influenza vaccines 4 weeks apart should be administered during the current season		
	One dose 0.25 ml of influenza vaccine should be administrated during the current season		
11	Indicate medications recommended for influenza prophylaxis and therapy:	119 (24.0)	376 (76.0)
	Inosine pranobex		
	Amantadine		
	Oseltamivir		
	Zanamivir		
	Acyclovir		
	Rimantadine		
12	Indicate correct statements about use of neuraminidase inhibitors in treatment of high risk patients:	312 (63.0)	183 (37.0)
	Treatment should be started as soon as the complications appear and be continued for 5 days		
	Treatment should be started as soon as influenza is suspected and be continued for 5 days		
	Treatment should be started as soon as laboratory confirmation of influenza appears and continued until the symptoms resolve		
	Neuraminidase inhibitors are recommended only for hospitalized patients		

(continued)

Table 2 (continued)

Items	(Multiple choice questionnaire; correct answers are underlined)	Correct answers *n* (%)	Incorrect answers *n* (%)
13	Influenza can be a self-limiting or progressive disease. Indicate symptoms of progressive influenza:	48 (9.7)	447 (90.3)
	Fever >40 °C		
	Chest pain, breathing effort in children		
	Impaired consciousness		
	Exacerbation of a chronic disease		
14	Indicate possible complications of influenza in pregnant women:	51 (10.3)	444 (89.7)
	Pneumonia		
	Respiratory failure		
	Stillbirth		
	Preterm birth		
	Newborn being small for gestational age		
	Heart defect in newborn		
15	Indicate factors influencing the interpretation of a rapid flu test:	69 (14.0)	426 (86.0)
	Patient age		
	Duration of symptoms		
	Disease prevalence in population		
	Biological sample type		
16	Indicate the methods of influenza diagnosis:	60 (12.1)	435 (87.9)
	Immunofluorescence tests		
	RT-PCR method		
	Viral culture		
	Serological tests		
	Rapid immunochromatographic assay (rapid test)		
17	Please indicate the possible routes of influenza vaccination:	56 (11.3)	439 (88.7)
	Intramuscular		
	Subcutaneus		
	Intradermal		
	Intranasal		
	Oral		
18	Indicate correct statements about co-infection of influenza with other pathogens:	133 (26.9)	362 (73.1)
	Influenza virus 'facilitates' pneumococcal infections		
	Influenza virus 'facilitates' meningococcal infections		
	Influenza virus 'facilitates' respiratory tract infections with other pathogens		
	Co-infection with influenza type A and B viruses is possible		

CDC Centers for Disease Control and Prevention, *WHO* World Health Organization

show that influenza is now the most dangerous infectious disease and one of the most significant threats to public health. The US ACIP, the European CDC, and the WHO recommend vaccination as the most effective preventive measure for seasonal influenza and as the first-line intervention to control the impact of seasonal influenza on public health (Grohskopf et al. 2016; WHO 2016b; European Commission 2014; Council of the European Communities 2009). The Polish National Immunization Program recommends the annual influenza vaccination for all individuals without medical contraindications who are 6 months of age and older. High-risk individuals, their close contacts, and healthcare workers remain the high-priority

Table 3 Influenza-related morbidity and mortality in Poland in recent epidemic seasons

Influenza season	[a]Morbidity; *n*	[a]Hospitalizations; *n*	Percent of hospitalizations	Incidence per 100,000	Deaths; *n*
2014/15	2,788,911 (+43%)	9,013 (+40%)	0.32%	7339	10
2015/16	3,070,082 (+10%)	12,309 (+37%)	0.40%	8079	111
2016/17	3,793,770 (+24%)	13,779 (+12%)	0.36%	9842	24

[a]Percent of increase from the preceding season in parenthesis. Data preceding the 2014/15 season not shown

target groups for immunization. Despite clear and broad recommendations, the percentage of individuals vaccinated against influenza in the general population has been highly unsatisfactory, ranging from 2 to 5% in 2005–2014 (Czarkowski et al. 2015). One of the underlying reasons is that vaccines have become not only a domain of medical knowledge but also a subject of a heated public debate in recent years. The anti-vaccination movements and the popularization of pseudo-scientific contents on the Internet seem to significantly influence both medical professionals' opinions and the decision-making process concerning immunization. The main inclination observed is to stoke fear and overstate the side effects of vaccines while to understate the risk of vaccine-preventable diseases and, in extreme cases, denying their existence (NWO Report 2017; Verger et al. 2016; BeWellBuzz 2015; Healy et al. 2014). In 2011, the WHO definition of vaccine hesitancy was coined as 'delay in the acceptance or refusal of vaccines despite the availability of vaccination services' and a working group on vaccine hesitancy has been established (WHO 2014, 2016a). Vaccine hesitancy is a complex and emerging global problem that requires local monitoring. In Poland, the number of people questioning (negating) mandatory vaccinations in Poland tripled in 2011–2014.

The impact of vaccine hesitancy on the vaccination rate has been observed in many countries and noted with concern by the Strategic Advisory Group of Experts on Immunization. The present survey revealed three main problems. Firstly, the unsatisfactory level of healthcare professionals' knowledge of influenza and influenza immunizations, which can be partly explained by the information glut that it is difficult to navigate through to draw a sensible meaning, a deluge of highly-specialist information, and other, not strictly factual but time-consuming activities, e.g., frequently changing reimbursement rules, healthcare professionals should be aware of. The insufficient knowledge of vaccination against influenza among healthcare professionals is a phenomenon present in numerous countries, being confirmed by a low vaccination rate that remains persistently lower than the population target of 75% (Hulo et al. 2017; Newcombe et al. 2016; Nutman and Yoeli 2016; Kassianos 2015; Castilla et al. 2013; Opstelten et al. 2008). Secondly, there appears a striking disproportion between the good knowledge of healthcare professionals on contraindications to vaccination and poor knowledge on indications to immunization, influenza complications, and the risk groups. Undoubtedly, this incomplete knowledge contributes to the low vaccination rate against influenza in Poland. Lastly, there is an issue of the universal trivialization of influenza infection. As a result, healthcare professionals are not well aware of symptoms pointing to the possible presence of a severe disease or of poor prognostic factors. General practitioners mostly see ambulatory patients with a benign disease course. Their personal experience may tame the true perception of influenza severity and burden, and may result in disease trivialization, often confused with a common cold. The present study also showed that most respondents could not properly define the risk groups, the signs and symptoms of severe influenza, and the predictors of its complications. In all likelihood, this is also reflected in a low vaccination rate specifically concerning the healthcare professionals in Poland, amounting to barely 6% in 2007/08 and 9% in the most recent 2016/17 season. A number of studies have examined the decision-making process

regarding vaccinations. The results indicate that one of the most important factors influencing one's decision to become vaccinated is the attitude of the physician providing advice to the patient (Arriola et al. 2015; WHO 2014; Leask et al. 2012; Cooper et al. 2008; Schmitt et al. 2007). Healthcare professionals' knowledge and attitudes about vaccines determine the intention to recommend the vaccine to patients and thus also vaccine uptake by patients (Nessler et al. 2014; Flicoteaux et al. 2014; Clark et al. 2009; Hollmeyer et al. 2009; Posfay-Barbe et al. 2005).

We believe the present study has identified the essential reason for a low influenza vaccination rate in Poland, which is the poor knowledge of a disease threat, with the undue perception of contraindications to immunization and an excessive fear of adverse effects. In 2016, European recommendations concerning the diagnosis and prevention of seasonal influenza were 'harmonized to better identify influenza outbreaks and to move towards reaching the target vaccination rate of 75% throughout Europe' (Kassianos et al. 2016). Hopefully, the harmonizing of recommendations will facilitate the assimilation of knowledge, but it cannot substitute for the continuing medical education.

5 Conclusions

The study identified the most important reasons for a low influenza vaccination rate in Poland, which are the disease trivialization and unsatisfactory knowledge among healthcare professionals of influenza and influenza immunization, combined with the perception of an exaggerated relevance of side effects of, and contraindications to, vaccination. All healthcare workers should be provided with continuous education programs focused on influenza complications, poor prognostic factors, risk groups, and the indications for vaccination. Subspecialists should be educated that influenza can exacerbate diseases in their field of specialization, which can be prevented with a vaccine.

Conflicts of interest The authors declare no conflicts of interest in relation to this article.

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Benign Acute Childhood Myositis During Influenza B Outbreak

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Abstract

Benign acute childhood myositis (BACM) is a syndrome classically occurring in children during the convalescent phase from a febrile upper respiratory tract infection, most commonly after influenza B. BACM can cause difficulty walking due to severe calf pain. Laboratory results show increased serum creatinine kinase and AST. Although alarming, BACM is self-limiting with symptoms disappearing within a week. Herein, we described a case series of BCAM in children in two cities in Poland during the influenza outbreaks in 2012/2013 and 2014/2015. We discussed the presentation and the clinical workup and examinations of the myositic syndrome. In addition, we evaluated the association of BACM with influenza B. We detected specific IgG against influenza B virus in 83% of the children diagnosed with BCAM. Reports from the National Institute of Public Health – National Institute of Hygiene in Warsaw, Poland confirmed a high rate of influenza B cases during both epidemic seasons in question.

Keywords

Children • IgG immunoglobulins • Influenza infection • Myalgia • Immunization

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1 Introduction

1.1 Benign Acute Childhood Myositis

Benign acute childhood myositis (BACM) was first described as 'Myalgia Cruris Epidemica' by the Swedish physician Lundberg in 1957. The disease is characterized by sudden onset of calf pain, less frequently by pain in the thighs or neck, which causes difficulty walking. Two characteristic gaits are noted: toe walking or a wide-based stiff-legged gait. Unilateral pain, abnormal results of neurological examinations, muscle weakness, and failure to improve after 4 days excludes the diagnosis of BACM. Typically, BACM develops during the convalescent phase of a febrile upper respiratory tract infection, most commonly after primary infection with influenza B. It occasionally occurs after parainfluenza, influenza A, RSV, *Mycoplasma pneumoniae*, adenovirus, enterovirus, or dengue virus infections. It mainly affects boys between 6 and 9 years of age, but BACM can also occur among adults, although less frequently. Laboratory investigations show increased serum creatinine kinase and aminotransferase activities (AST) and leucopenia. Urinalysis and creatinine are normal. The pathogenesis of BACM is unclear. Muscle biopsies performed during myositis show a focal fiber vacuolization and extensive necrosis, mostly with a little inflammatory involvement. No immunoglobulin or complement deposits, pointing to an autoimmune character of the ailment are observed. Ample data suggest a direct muscle damage due to viral invasion mainly related to influenza B (Mall et al. 2011; Panghaal et al. 2008). Although potentially alarming, BACM is a self-limiting condition with symptoms disappearing within a week. Neuraminidase inhibitors are not recommended due to the occurrence of BACM in the late phase of the influenza. No treatment other than analgesia is required.

1.2 Influenza B

Influenza is an acute febrile disease of the respiratory tract caused by influenza A or B viruses. Although self-limiting and usually uncomplicated in healthy children, due to a large number of cases during outbreaks, influenza causes high morbidity and a high number of hospitalizations annually, especially among infants and children with chronic health conditions. The clinical presentation of influenza in children is variable. Typical symptoms include fever, headache, cough, myalgia, and malaise. These symptoms are often accompanied by a sore throat and rhinitis. Children with uncomplicated influenza usually improve over 2–5 days, although the illness may last for a week or longer. The most common influenza complication in children is otitis media, occurring in 10–50% of children, but more serious complications related to a lower respiratory tract involvement, such as laryngotracheitis, bronchitis, bronchiolitis, and pneumonia, also are common. Preexisting chronic neurologic diseases pose a strong threat of influenza-related complications (Havers et al. 2014). Influenza may exacerbate chronic respiratory diseases, especially asthma (Glezen 2000). Other significant complications of influenza include myocarditis, pericarditis, toxic shock syndrome, and Reye's syndrome. Influenza also is associated with neurologic complications in healthy children (2–8%). Febrile seizures are the most common; the second is encephalopathy. Other rare neurologic complications include stroke, focal neurologic deficits, Guillain-Barré syndrome, acute disseminated encephalomyelitis (ADEM), transverse myelitis, and benign acute childhood myositis (BACM) that has an excellent prognosis and is frequently related to influenza B (Mall et al. 2011; Panghaal et al. 2008). Although influenza B is considered be more benign, clinical symptoms of influenza A and B are similar, and the virus type cannot be determined based solely on the clinical criteria. One significant

difference is the more common occurrence of severe muscle pain due to myositis in the influenza B infection, which is very rare in influenza A (Hu et al. 2004). Compared to influenza B patients, influenza A patients present more frequently with rhinitis, cough, and expectoration and less frequently with gastrointestinal symptoms (Mosnier et al. 2015).

One hypothesis explains differences in the rate of myositis as sequela of influenza A and B due to a different viral structure. Segment 6 of the influenza B virus genome encodes a small glycoprotein (NB) that is an integral component of the membrane of the influenza B virion and may play a role in virus entry making influenza B more myotropic than influenza A (Hu et al. 2004). Two antigenically distinct lineages of influenza B: Victoria-like strains and Yamagata-like strains have been circulating globally since 1985. The antibody response to influenza B infection in children is lineage-specific, with no cross-reactivity between lineages. The lineage causing BACM has not yet been established.

The annual influenza vaccination is the most effective way to prevent influenza. The vaccine should be administered each year before the influenza season begins. Influenza immunization is recommended for everyone older than 6 months of age. In Poland, vaccination against influenza is recommended, but not reimbursed. Parents who want to vaccinate their children must pay for the vaccine, which is likely one of the reasons that vaccination coverage is very low, amounting to 6–9% in the general population and only 3% in children (Kuchar et al. 2013). The trivalent seasonal influenza vaccines contain three strains, one from each A subtype (H1N1 and H3N2) and one type B virus. Trivalent vaccines for the 2015/2016 season contained A/California/7/2009 (H1N1)pdm09-like virus, A/Switzerland/9715293/2013 (H3N2)-like virus, and B/Phuket/3073/2013-like virus. Due to a limited cross-reactivity of lineage-specific antibodies against influenza B induced in response to influenza vaccination, its efficacy could be easily improved by the inclusion of both influenza B lineages to produce quadrivalent formulations representing a next logical step for seasonal influenza vaccines (Ambrose and Levin 2012).

The aim of the present study was to describe a case series of BCAM during the influenza outbreaks in 2012/2013 and 2014/2015 and to evaluate the association of BACM with influenza B.

2 Methods

Due to a retrospective nature of the study, a local Ethics Committee of Wroclaw Medical University in Poland waived the requirement of obtaining ethical clearance. We conducted a retrospective audit of the medical records of the children diagnosed with benign acute childhood myositis admitted to the pediatric ward at the Regional Medical Center in Opole and the Department of Pediatric Infectious Diseases in Wroclaw during the influenza epidemic seasons of 2012/2013 and 2014/2015. There were 13 such patients in total; 12 children were hospitalized in Opole in March and April of 2013 and one child in Wroclaw in February of 2015. The patients' median age was 6 years, range 2–12 years. Eight of them were boys (62%). We evaluated the symptoms on admission and the following laboratory results: white blood cell counts (WBC), serum creatinine kinase activity (CPK), C-reactive protein (CRP), serum aspartate aminotransferase (AST), and alanine aminotransferase (ALT). Age, gender, vaccination history, and previous treatment also were collected.

The association between BACM and influenza B was assessed with a Euroimmun Anti-Influenza B IgG test (Euroimmun AG; Lübeck, Germany). The test was performed in the 12 patients diagnosed with BACM in the city of Opole. These results were compared with those obtained in the control group consisting of 29 age- and gender-matched children hospitalized for other non-infectious reasons in the same seasons and region.

Statistical elaboration was performed using Statistica v12 for Windows (StatSoft; Tulsa, OK).

3 Results

Thirteen children were admitted to the pediatric wards in Wroclaw and Opole during two consecutive influenza seasons because of acute onset of muscle pain preceded by fever. Eight children (62%) complained of severe calf pain and 6 children presented with an altered gait (46%). Muscle pain developed within 2–4 days after onset of an upper respiratory tract infection and fever. None of the children reported a history of similar muscle pains in the past. During the physical examination, beside muscle pain, symptoms of the upper respiratory infection were observed, such as non-productive cough, sore throat, and nasal discharge. Muscle strength was difficult to test because of a pain-related discomfort. Neurologic examinations were normal in all patients. Laboratory findings showed the increased CPK in 10/13 (77%) patients (median of 1202 U/L; range from 378 to 5784 IU/L), increased AST in about half of the patients (7/13; 54%) (median 110 U/L; range from 66 to 214 IU/L), and leukopenia in 4/13 (31%) patients. ALT was normal in almost all patients and CRP was normal in the vast majority of patients (11/13; 85%). Muscle pain persisted for an average of 3 days; while the median stay in the hospital was 2 days. None of the patients were vaccinated against influenza or treated with antivirals, including neuraminidase inhibitors.

The patient details are summarized in Table 1.

The results of serological testing in the 12 BACM patients from Opole and in the control group composed of children hospitalized for other reasons are presented in Table 2. We detected specific IgG against influenza B virus in 10/12 (83%) children diagnosed with BCAM *vs.* 9/29 (31%) in the control group. The geometric mean antibody concentration in the children with positive IgG against influenza B was

Table 1 Characteristics of 13 children with benign acute childhood myositis

	Age (years)	Gender	CPK (IU/L)	AST (U/L)	Altered gait	City	Anti-influenza B IgG[a]
1	6	Male	1212	71	no	Opole	Positive
2	2	Male	639	Not tested	no	Opole	Positive
3	8	Female	5784	185	yes	Opole	Positive
4	6	Male	1193	66	yes	Opole	Positive
5	7	Male	1727	81	yes	Opole	Positive
6	6	Female	378	39	no	Opole	Positive
7	6	Male	2282	110	no	Opole	Positive
8	5	Male	41	26	no	Opole	Negative
9	8	Male	488	214	yes	Opole	Negative
10	6	Female	630	Not tested	yes	Opole	Positive
11	3	Female	81	37	no	Opole	Positive
12	12	Female	149	21	No	Opole	Positive
13	7	Male	4767	128	Yes	Wroclaw	Not tested

[a]Euroimmun serological test

Table 2 Anti-influenza B IgG test in children diagnosed with BACM and in the control group

	BACM group	Control group	p-value
No. of patients	12	29	–
No. of females (%)	5 (41%)	15 (51%)	NS
Median age (years)	6	6	NS
No. of positive anti-infuleza B IgG tests (%)[a]	**10 (83%)**	**9 (31%)**	**0.02**

[a]Euroimmun serological test

similar in both groups; 94.8 *vs.* 82.8 RU/ml (relative units), in the BCAM and control group, respectively.

4 Discussion

Sporadic cases of BACM regularly occur during an influenza season and occasionally BACM occurs as a large outbreak. Since its first description, BACM has been reported in the literature mostly as case reports. To our knowledge, about fifteen BACM outbreaks have been described over the last 50 years, for instance, 40 children due to dengue virus in India in 2001/2002 (Rajajee et al. 2005), 219 children in Germany in 2007/2008 due to influenza B virus (Mall et al. 2011), and 49 children in Switzerland also due influenza B virus (Ferrarini et al. 2014). The characteristics of our patients such as the median age of 6 years and the predominance of males are consistent with results reported in those other studies. The cause of male predominance is unclear, but it could be related to a greater physical activity or genetic predisposition of male gender. Clinical presentation has been similar across all previous (Ferrarini et al. 2014; Mall et al. 2011) and current report; nearly all patients had muscle pain preceded by fever. We noted a severe calf tenderness in most than half (62%) and altered gait in about half of the patients (46%). These rates were appreciable lower than those reported in other studies. For instance, all patients with BACM presented with calf pain and 93% refused to walk in a study in Saudi Arabia (Al-Qahtani et al. 2014). The difference might be explicable by unclear diagnostic criteria used in various reports. Myalgia in the prodromal phase of influenza is usually generalized and diffuse, which differentiates it from the calf pain in BCAM. As far as biochemical examinations are considered, most of the patients in the present study (77%) had an increased CPK activity (median of 1202 IU/L) and half of them (54%) had elevated AST. CRP was within the normal range in the majority of the patients (85%), while white blood cell count was normal in 54% of them. Leukopenia was noted in 31% of patients. These results are consistent with those from other studies (Al-Qahtani et al. 2014; Ferrarini et al. 2014; Mall et al. 2011). The serological presence of antibodies against influenza B virus in the control subjects, who had different non-infectious ailments, also was consistent with the literature findings. In a German study, the overall prevalence of these antibodies in the entire pediatric population has been 47.0%, with the mean antibody concentration of 54.7 RU/ml, in the period of 2008–2010 (Sauerbrei et al. 2014). We believe that the BACM cases currently described were caused mainly by influenza B for several reasons. Firstly, we detected IgG against influenza B in 10 out of 12 patients with BCAM, which was significantly more than in the control group. Secondly, the National Institute of Public Health – National Institute of Hygiene in Warsaw, Poland, the influenza monitoring center for the country, confirmed a high rate of influenza B infections during both epidemic seasons in question; 20.4% and 47.0% for 2012/2013 and 2014/2015, respectively. Thirdly, none of our patients were vaccinated against influenza and influenza vaccine coverage in children in Poland is very low (approximately 3%) (Kuchar et al. 2013). Our reasoning pointing to the plausible link between BACM and influenza B virus is congruous with the findings of other studies where BACM also was most commonly related to influenza B virus (Ferrarini et al. 2014; Mall et al. 2011).

In conclusion, benign acute childhood myositis is a rare, self-limiting disease for which only analgesia is recommended. It usually occurs a few days after onset of classic symptoms of influenza, at the time when these symptoms start waning, which makes BACM difficult to diagnose. BACM can be of concern for parents and physicians who may not be much familiar with this disease. That may lead to unnecessary hospitalizations and the pain and hassle of clinical workup. Since most BACM cases seem to be related to influenza B in Poland, the introduction of a new quadrivalent influenza vaccine containing both lineages of influenza B could more effectively prevent the occurrence of BACM and of other influenza complications.

Conflicts of interest The authors declare no conflicts of interest in relation to this article.

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Serum Diamine Oxidase in Pseudoallergy in the Pediatric Population

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Abstract

Histamine intolerance (pseudoallergy) is a poorly investigated type of food hypersensitivity. The main enzyme responsible for histamine degradation in the extracellular matrix is diamine oxidase (DAO). Disturbances in the concentration or activity of DAO may lead to the development of clinical signs of allergy. The aim of the present work was to assess the DAO concentration, peripheral blood morphology, lymphocytes phenotyping (CD3+, CD4+, CD8+, CD19+, NK cells, NKT cells, and activated T-cells), and natural regulatory Treg (nTregs) cell population (CD4+, CD25+, CD127low, and FoxP3) in 34 pediatric patients with histamine-dependent syndromes. Patients were divided into two groups: classical allergy and pseudoallergy on the basis of IgE concentration. The investigation was based on the analysis of peripheral blood samples. A significantly lower serum DAO, both total and specific IgE, concentration was found in the pseudoallergy group compared with the allergy group. There were no significant differences in blood morphology or lymphocyte populations. A similar level of nTreg lymphocytes was also found in both groups, although it was lower than that present in healthy individuals. The findings suggest that the serum DAO is responsible for the symptoms of histamine intolerance. Moreover, a general decrease in nTreg cells in comparison with healthy individuals may lead to symptom aggravation.

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Keywords
Allergy • Angioedema • Dermal lesions • Headache • Immunological disorders • Stomachache

1 Introduction

Histamine intolerance is a poorly understood type of food hypersensitivity mimicking an allergic reaction. It is referred to as pseudoallergy. In both histamine intolerance and allergy, the factor responsible for the development of symptoms is histamine (β-imidazolyl-4-ethylamine), acting on numerous receptors present in various cells. Histamine-dependent symptoms develop in the alimentary system (stomachache, diarrhea, nausea, and flatulence), skin (rashes, angioedema, reddening, and itching), cardiovascular system (arrhythmias and hypotension), nervous system (headaches and fainting), and in other organs (Karpińska-Gasztoł et al. 2014; Millichap and Yee 2003).

In case of allergy, immunological reaction triggers a release of histamine into the blood. The reaction involves the IgE cross-linking on mast cells or basophiles following an allergen provocation, leading to a rapid release of histamine from those cells (Maintz et al. 2006). A trace amount of an allergen is sufficient to evoke the reaction. In case of histamine intolerance, a non-immunological pathway leads to the accumulation of histamine in excess of the body tolerance threshold due to an excessive ingestion of histamine-rich food with insufficient mechanisms of its degradation or, to a lesser extent, due to a number of factors triggering a release of histamine from cellular reserves (Table 1) (Smolinska et al. 2014; Czerwionka-Szaflarska and Zielińska-Duda 2009).

There are only two pathways of histamine metabolism in humans (San Mauro Martin et al. 2016; Górski 2007). The principal enzyme responsible for histamine degradation in the extracellular space and for preventing its excessive absorption is diamine oxidase (DAO) (Schwelberger et al. 2013, Maintz et al. 2006). Disorders of histamine degradation by DAO may result from the enzyme deficiency or its impaired function due to the action of pharmaceuticals, intestinal flora disorders, inflammatory diseases, and malignancies of the alimentary tract (Table 2) (Skypala et al. 2015; Maintz and Novak 2007). Another enzyme capable of degrading histamine, N-methyltransferase, acts intracellularly and plays no significant role in the metabolism of histamine supplied with food. The role of N-methyltransferase in the diagnostic and therapeutic procedures involving histamine intolerance seems negligible, despite its presence in various cells, including the respiratory epithelial cells.

When histamine-dependent symptoms are present, the diagnostics focus on confirming the allergic background of the ailment. The guidelines have been developed, along with the interpretation of readily available diagnostic methods, including the determination of total IgE and specific IgE and skin-prick tests (Høst et al. 2003). New diagnostic tools are also under development such as the determination of platelet-activating factor or mastocytic tryptase level (Vadas 2016; Vitte 2015; Sala-Cunill and Cardona 2015; Lieberman 2014; Akin et al. 2007). On the other hand, there is striking lack of a reliable diagnostic tool in case of a suspected histamine intolerance.

Demarcation between histamine intolerance and allergy is a challenge. Frequently, patients presenting histamine-dependent symptoms are classified as allergic, even though the allergic background has not been confirmed. This misinterpretation is associated with lasting consequences, particularly in children. The pseudoallergy is often suspected subsequent to a long-term observation in patients who exhibit at least two histamine-dependent symptoms that improve after a 4-week low-histamine diet or

Table 1 Food products high in histamine content and histamine releasing

Products high in histamine	Products stimulating histamine release
Alcoholic beverages (red wine, red, beer, champagne)	Alcoholic beverages (wine, beer)
Fish, including marinated, smoked, tinned	Marinated, salted and smoked fish
Seafood (crabs, oysters, caviar, mussels)	Tinned meat
Red meat and highly processed meat products (pepperoni, salami), tinned meat, offal	Milk and dairy products
Fermented dairy products	Certain herbs (mint, chamomile, linden, sage, pansy, willow)
Mushrooms	Mushrooms
Cheese (processed, mouldy, hard and semi-hard)	Pickled vegetables
Preserved and pickled vegetables	Certain vegetables (spinach, pepper, lettuce, potato, tomato, cucumber, peas, radish, courgette, leek, corn, carrot, beetroot)
Certain vegetables (beans, peas, lentils, spinach, lettuce, tomato, cabbage)	Certain fruits (berries, currants, citrus fruits, kiwi fruit, apple, plums, cherries, banana, melon, watermelon, pineapple, peach, grapes, apricot)
Certain fruits (berries, plums, grapes, avocado, pineapple, citrus fruits)	Preserved and dried fruits (raisins, figs, apricot, plums)
Preserved and dried fruits (raisins)	Carbonated drinks, fruit and vegetable juice
Sweets (chocolate, jam, marzipan, nougat)	Sweets (chocolate, honey, caramel, nougat)
Drinks (coffee, tea, cola)	Vinegar, mayonnaise, ketchup, oils, olive oil
Vinegar, mayonnaise, ketchup	Cereal products, seeds and nuts (soy, peanuts, almonds)
Certain spices (curry) Cereal products and yeast-based products	Food additives (colorants, preservatives, antioxidants, flavor enhancers)

Table 2 Factors inhibiting diamine oxidase (DAO) activity

DAO inhibitors
H_2 receptor antagonists (e.g., cimetidine)
Certain antibiotics (e.g., cefuroxime, clavulanic acid)
Antiarrhythmics (e.g., propafenon)
Antihypertensives (e.g., verapamil)
Analgesics (e.g., morphine, metamizole)
Radiological contrast media
Anesthetics (e.g., thiopental)
Antispasmodics
Motility agents (e.g., metoclopramide)
Diuretics (e.g., amiloride)
Mucolytics and broncholytics (e.g. acetylocysteine, ambroxol, aminophyline)
Other factors that inhibit DAO activity
Intestinal microflora disorders (excessive intake of probiotics, chemotherapy)
Excessive intake of biogenic amines (tyramine, putrescine)
Gastrointestinal tract disorders (inflammatory diseases, neoplastic diseases)

antihistamine medications (Kovacova-Hanuskova et al. 2015; Weidenhiller et al. 2012).

It is estimated that approximately 1% of the general population presents symptoms of histamine intolerance caused by DAO deficiency (Jarisch 2004). However, there are no reliable publications indicating the ratio of those patients in the overall group presenting histamine-dependent symptoms, or frequency of histamine intolerance and allergies coinciding. There are also no in-depth reports focusing on the pediatric population. Therefore, the present study seeks to

define a link between reduced serum DAO level and histamine intolerance in children. Attention was drawn to the plausible association of histamine intolerance with changes in peripheral blood cell counts and in subpopulations of regulator lymphocytes in a hope of identifying a reliable and rapid method enabling the diagnosis of histamine-dependent symptoms.

2 Methods

2.1 Patients

The study was approved by the Bioethics Committee of the Military Institute of Medicine in Warsaw, Poland (permit no. 141/16). The parents of qualified children gave written informed consent. Thirty four children with histamine-dependent symptoms were enrolled into the study. They were divide into two groups: allergy group (inclusion criteria: high concentration of total IgE or specific IgE compared to the age-matched healthy population) – 26 patients, aged 7 months to 17 years, and the pseudoallergy group (inclusion criteria: low concentration of total IgE or specific IgE compared to the age-matched healthy population) – 8 patients, aged 18 month to 11 years. Standard diagnostic interviews were conducted to assess the histamine-dependent symptoms and the probability of allergy. Patients or parents filled out the questionnaire by ticking yes or no answer. The results were shown as a percentage of positive answers.

2.2 Hematological Investigation

The blood, 500 μl taken into EDTA tubes, was investigated using the hematology systems (Sysmex XN 1000, Kobe, Japan and Advia 2102i, Siemens Healthcare GmbH, Erlangen, Germany) to determine the following parameters: white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW%), red cell distribution width absolute (RDW-a), platelets (PLT), and mean platelet volume (MPV).

The determination of WBC phenotype was performed as described previously (Kalicki et al. 2013). Briefly, cells in 100 μl of whole blood were labeled with the appropriate antibodies in the dark for 20 min (BD Simultest™ – IMK Plus Kit, BD Biosciences, Warsaw, Poland). Next, erythrocyte lysis was performed in room temperature for 10 min (FACS Lysing Solution, BD Biosciences). Afterward, the cells were washed twice with 2 ml of phosphate buffer solution (PBS) and fixed in 200 μl of 1% v/v paraformaldehyde/PBS solution. Phenotypic analysis was performed with flow cytometry (FACS Calibur; BD Biosciences) and analyzed with CellQuest Pro software (BD Biosciences). Three thousand counts of lymphocytes finished the acquisition. Results were expressed as means ±SE%.

The determination of nTreg cell population and gating restriction were performed as described previously (Lipińska-Opałka et al. 2017). Briefly, the cells in 100 μl of whole blood were stained with primary antibodies CD4-PerCP, CD25-APC, and CD127-FITC (extracellular staining; BD Bioscence, Warsaw, Poland) or with the appropriate isotype control with the addition of ally CD4-PerCP antibody. After 20 min, erythrocyte lysis was performed (FACS Lysing Solution, BD Biosciences) followed by fixation and permeabilization (Fixaton/Permeabilization buffer BD Pharmingen, Warsaw, Poland). The cells were then stained with FoxP3 PE or isotype IgG1 kappa PE antibody for 45 min in the dark in room temperature. The cells were acquired in flow cytometry. Ten thousand counts of CD 4 PerCP positive cells finished the acquisition. Results were analyzed by CellQuest Pro software (BD Biosciences) and expressed as means ±SE %.

2.3 Biochemical Investigations

Total IgE concentration was determined in the serum samples using a solid-phase enzyme-linked immunosorbent assay (Sandwich ELISA), calibrated with commercially available IgE standards. DAO content was measured in the serum samples using an ELISA assay kit for D-amino acid oxidase (Cloud-Clone Corp, Katy, TX). Results were expressed as means ±SE IU/ml.

2.4 Statistical Elaboration

Data distribution was evaluated using the Shapiro-Wilk test. Differences between the allergy and psudoallergy groups were evaluated with Student's *t*-test or Mann-Whitney U test as required. A p-value <0.05 defined the statistically significant changes. The analyses were performed with a commercial GraphPad Prism v5 software package (GraphPad Software, La Jolla, CA).

3 Results

3.1 Allergy vs. Pseudoallergy

The main criterion for the differentiation of allergic from pseudoallergic children was the serum concentration of IgE immunoglobulin. The difference in the total IgE content was outstanding (Fig. 1), enabling further elaboration of clinical aspects in clearly demarcated groups of children.

3.2 Symptoms

Generally, there were no significant differences in the manifestation of specific histamine-dependent systemic symptoms in the children with allergy and pseudoallergy. However, there was a tendency for a lower incidence of skin symptoms in children with pseudoallergy. We also observed the appearance of symptoms after ingestion of food nearly twice as often in children with pseudoallergy (Table 3).

3.3 Serum DAO Concentration

In the group of patients with allergy, the mean serum DAO concentration amounted to 83.01 ± 6.73 IU/ml. In the pseudoallergic group, the mean DAO concentration was significantly lower 46.40 ± 7.19 IU/ml ($p = 0.0016$) (Fig. 2).

There were no significant differences in the majority of blood morphological indices in the children with allergy and pseudoallergy. The notable exceptions were a significantly greater absolute and relative levels of eosinophils in the allergy than pseudoallergy group and somewhat smaller mean corpuscular volume and concentration of hemoglobin in the latter group (Table 4).

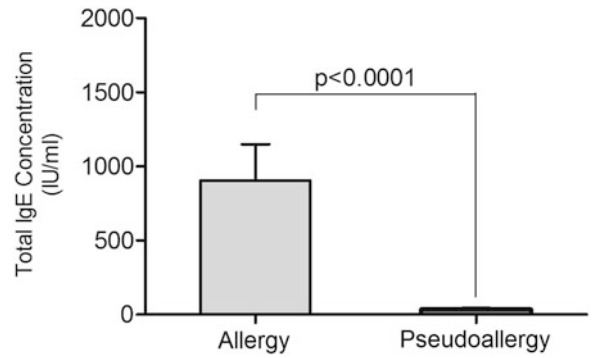

Fig. 1 Serum total IgE concentration; data are means ±SE

Table 3 Patient symptoms (% of cases)

	Allergy	Pseudoallergy
Skin (atopic dermatitis, pruritus, urticaria, erythema)	76.9%	50.0%
Neural system (pain and dizziness, nausea)	7.4%	0%
Digestive system (diarrhea, bloating, vomiting, lips and tongue swelling)	22.2%	25.0%
Respiratory system (runny nose, sneezing, wheezing, dyspnea)	70.4%	75.0%
Circulatory system (arrhythmias, syncope, dizziness)	0%	0%
Seasonality	29.6%	12.5%
Symptoms appearing after food ingestion	22.2%	50.0%
Symptoms appearing after drug use	3.7%	12.5%

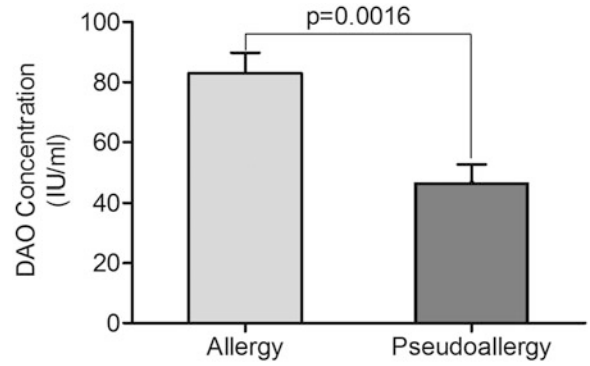

Fig. 2 Serum DAO concentration; data are means ±SE

In both allergy and pseudo-allergy groups, there were no significant differences in the percentage of the following lymphocyte subpopulations: B-cells, T-cells, CD4, CD8, CD4/8 ratio, NK cells, NKT cells, and activated T-cells (Fig. 3). Nor were there any significant differences between the percentages of nTregs (CD4+, CD25+, CD 127low, and FoxP3+).

4 Discussion

Attempts to examine the role of histamine and reduced serum DAO activity in the diagnostics of food intolerance have been made in the 1980s and 1990s. Provocation tests, intestinal biopsy, and a determination of serum DAO following heparin injections were the procedures of choice at that time; which, however, were apt to produce numerous adverse effects (Wantke et al. 1994; Wantke et al. 1993; Lessof et al. 1990).

New methods for diagnosing pseudoallergy are currently sought. Mušič et al. (2013) have examined a correlation between serum DAO activity in patients with suspected histamine intolerance and the effect of a low-histamine diet in a group of 316 adults. A similar study has been conducted by Manzotti et al. (2016) in 14 adults presenting histamine-dependent symptoms, following the exclusion of the allergic background of observed symptoms and of factors leading to the enzyme dysfunction. Both studies confirm the existence of a correlation between reduced DAO activity and the development of histamine intolerance. There are reports suggesting the applicability of skin-prick tests with histamine-soaked flakes as a diagnostic method (Kofler et al. 2011).

Clinical studies on histamine intolerance in children are scarce or involve small groups of patients (Hoffmann et al. 2013; Rosell-Camps et al. 2013; Millichap and Yee 2003). Therefore, there are no reliable data determining a correlation between DAO concentration and histamine intolerance symptoms; not to mention the

Table 4 Blood hematological indices

	Allergy	Pseudoallergy	*p*-value
WBC# (×10^9/l)	7.5 ± 0.4	8.3 ± 0.9	0.778
RBC# (×10^12/l)	4.8 ± 0.1	4.9 ± 0.1	0.298
HGB (g/dl)	13.0 ± 0.2	12.7 ± 0.2	0.224
HCT%	38.6 ± 0.6	37.9 ± 0.7	0.755
MCV (fl)	80.2 ± 0.6	77.9 ± 0.8	0.062
MCH (pg)	27.2 ± 0.2	26.0 ± 0.3	**0.004**
MCHC (g/dl)	33.9 ± 0.2	33.4 ± 0.3	**0.009**
PLT# (×10^9/l)	313.4 ± 17.1	343.7 ± 23.4	0.348
MPV (fl)	8.5 ± 0.3	8.0 ± 0.8	0.898
PCT%	0.26 ± 0.02	0.27 ± 0.03	0.439
PDW (fl)	10.7 ±1.4	13.1 ±1.5	0.316
PDW%	26.6 ±5.1	32.8 ±8.4	0.741
RDW-SD (fl)	36.3 ±0.5	36.4 ±1.2	0.965
RDW-CV%	12.7 ±0.1	12.9 ±0.5	0.584
LUC# (×10^3/μl)	0.12 ±0.03	0.17 ±0.05	0.528
LUC%	1.6 ±0.3	2.3 ±0.6	0.800
LY#	3.1 ±0.2	4.0 ±0.9	0.546
LY%	42.6 ±1.7	45.2 ±5.1	0.671
NE# (×10^3/μl)	3.2 ±0.2	3.4 ±0.5	0.919
NE%	42.8 ±1.8	43.1 ±4.9	0.966
MO# (×10^3/μl)	0.52 ±0.04	0.51 ±0.06	0.809
MO%	6.9 ±0.4	6.4 ±0.7	0.842
EO# (×10^3/μl)	0.43 ±0.06	0.22 ±0.03	**0.007**
EO%	5.6 ±0.7	2.8 ±0.4	**0.004**
BA# (×10^3/μl)	0.04 ± 0.0	0.04 ± 0.01	0.897
BA%	0.54 ± 0.04	0.57 ± 0.08	0.968
P-LCR (×10^3/μl)	21.0 ± 3.9	33.9 ± 6.5	0.144

Data are means ±SE; $p < 0.05$ denoting significant changes between the two groups in *bold*
WBC white blood cells, *RBC* red blood cell count, *HGB* hemoglobin, *HCT* hematocrit, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *PLT* platelet count, *MPV* mean platelet volume, *PCT* platelet crit, *PDW* platelet distribution width, *PDW%* relative platelet distribution width, *RDW-SD%* relative red blood cell distribution width, standard deviation, *RDW-CV%* relative red blood cell distribution width, coefficient of variation, *LUC#* large unstained cells – absolute content, *LUC%* large unstained cells – relative content, *LY#*,lymphocytes – absolute content, *LY%* lymphocytes – relative content, *NE#* neutrophils – absolute content, *NE%* neutrophils – relative content, *MON#* monocytes – absolute content, *MON%* monocytes – relative content, *EO#* eosinophils –absolute content, *EO%* eosinophils – relative content, *BA#* basophils –absolute content, *BA%* basophils – relative content, P-*LCR* ratio of large platelets

influence on the results of patients' age which could also factor in the present findings.

The present findings demonstrate that in addition to the known correlation between the development of pseudoallergy symptoms and reduced DAO activity, a similar correlation exists for the reduced serum DAO content. The correlation is present in the pediatric population, which suggests the use of a serum DAO measurement as a diagnostic tool in suspected cases of pseudoallergy in children. Moreover, a significantly greater ratio of eosinophils in the peripheral blood was notable in the group classified as allergy. A statistically significant difference in the hemoglobin indices, MCH and MCHC, could be a result of a small size of the study group and its low differentiation. Although a similar level of nTregs (CD4+, CD25+, CD 127low, and FoxP3) was found in both study groups, it was lower than that present in the healthy pediatric population (Lipińska-Opałka et al. 2017). This finding points to the plausibility of immunological dysfunction in both histamine intolerance and allergy. Further exploration of a

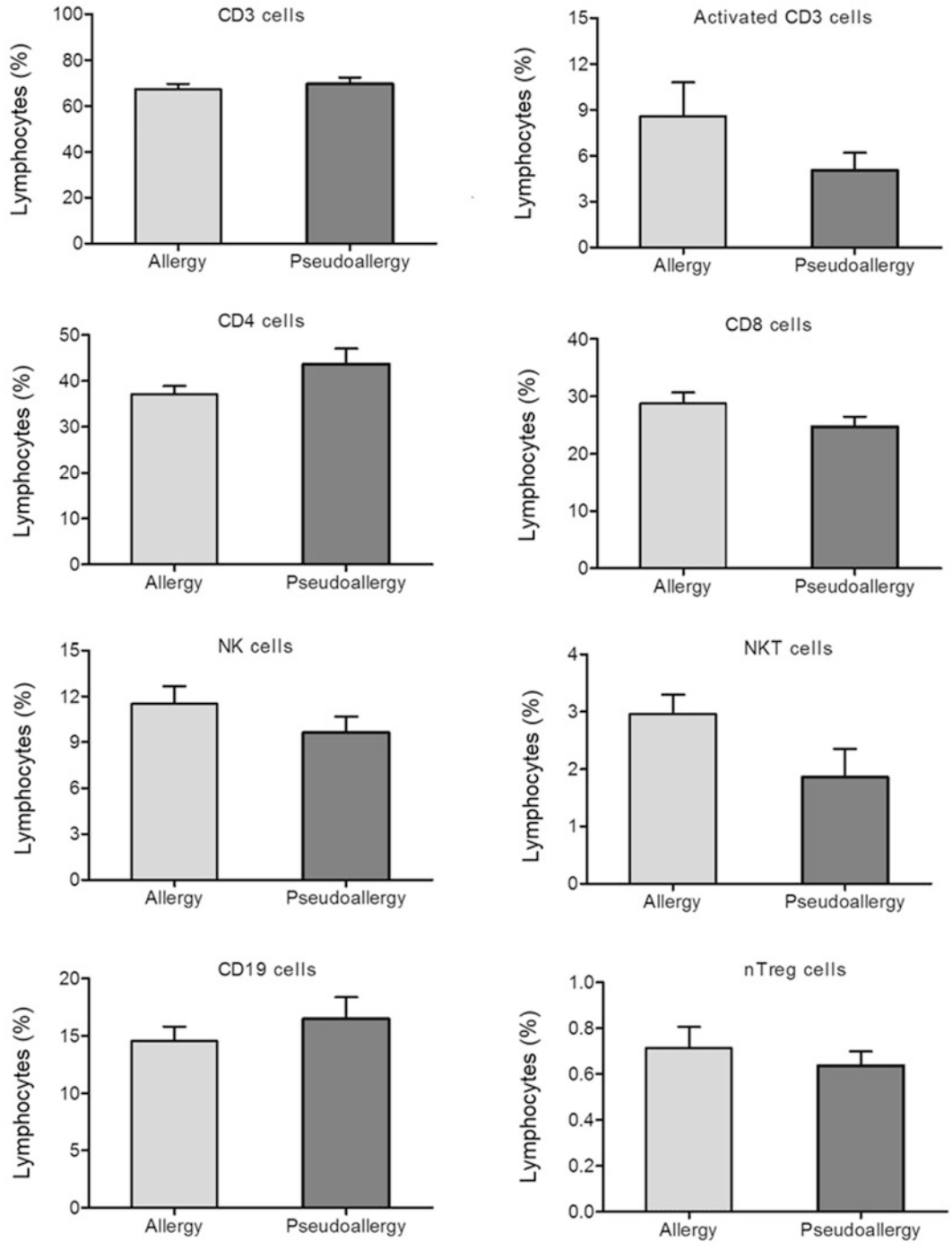

Fig. 3 Lymphocytes phenotyping in peripheral blood; data are means ± SE

link between serum DAO and regulatory lymphocytes is required to determine their mutual influence on the development of histamine dependent symptoms.

When considering the variability of pediatric organisms, a small sample size in the present study could interfere with the interpretation of results. Therefore, the study is a mere introduction to further explorations, including the determination of both serum concentration and activity of diamine oxidase in various age-groups.

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Spontaneous Elimination of Hepatitis C Virus Infection

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Abstract

Hepatitis C virus (HCV) is the etiological agent of chronic hepatitis C and a major cause of liver cirrhosis and hepatocellular carcinoma. Only a minority of infected individuals can clear the virus spontaneously. The knowledge of the determinants of virus clearance would allow the development of effective methods preventing its further spread and optimizing treatment regimens. Viral factors associated with spontaneous virus clearance in the acute phase of infection, such as HCV genotype, virus heterogeneity, and the impact of viral proteins on the immune system have been characterized. Likewise, host genetic markers, such as the interleukin genotypes, HLA alleles, and factors affecting the T lymphocyte response appear to play an important role. Studies have revealed that natural clearance of HCV infection in the chronic phase is rare and its mechanisms are not well understood. In this review, we present the state-of-the art knowledge on the viral and host factors affecting the spontaneous elimination of HCV infection.

Keywords

Genetic markers • HCV genetic heterogeneity • Hepatitis C virus • Host-related factors • Liver cirrhosis • Viral factors

1 Introduction

Hepatitis C virus (HCV) is the etiological agent of chronic hepatitis C and a major cause of liver cirrhosis and hepatocellular carcinoma. The role of HCV infection has been also documented in such concomitant diseases as mixed cryoglobulinemia, autoimmune diseases, B-cell

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non-Hodgkin's lymphoma, and glomerulonephritis (Jadali 2012). Therefore, increasing number of patients with HCV infection puts a substantial burden on the health system worldwide. The insight into the mechanisms of infection and factors associated with virus clearance would allow the development of effective methods preventing its further spread and optimizing the treatment regimens.

2 Epidemiology

Most recent studies show the HCV seroprevalence at 2.8%, which is more than 185 million infected individuals worldwide. It is believed that the majority of infections spread by blood transfusions, blood products, and intravenous drug administration. Based on the phylogenetic analysis of sequenced genomes, seven HCV genotypes and 67 subtypes are recognized. The global distribution of different genotypes and subtypes is also known. Some of them can be called epidemic since they are common across the world, such as genotypes 1a, 1b, 2a, and 3a, whereas others as endemic since they occur locally. The latter include genotype 4 in Central Africa and in the Middle East, genotype 5 in South Africa, and genotype 6 in Southeast Asia. It is estimated that genotype 1 is the most common worldwide and is responsible for 46.2% of HCV cases. The prevalence of genotype 3 is assessed to be 30.2%, genotypes 2, 4, and 6 are responsible for 22.8% of all cases, and genotype 5 comprises the remaining <1% (Messina et al. 2015).

It is estimated that 80–90% of HCV-infected patients develop chronic infection that is associated with liver-related morbidity and mortality. A minority of patients (10% to 46%) show the ability to spontaneously clear the virus within the time period as long as 3 years (Seaberg et al. 2015; Amini and Poustchi 2012; Grebely et al. 2006; Wiese et al. 2000). The clearance rate is 15% according to most clinical reviews (Micallef et al. 2006).

3 Factors Associated with Spontaneous HCV Elimination

Factors most likely associated with spontaneous virus clearance can be divided into two groups. One group consists of the virus-related factors that include the HCV genotype and a high rate of mutations and immune response suppression by viral antigens (Valva et al. 2014; Amini and Poustchi 2012). The other group represents the host-related factors such as race (Thomas et al. 2000), gender (Tillmann et al. 2010), age, concomitant infection with human immunodeficiency virus (HIV) (Seaberg et al. 2015), polymorphisms of interleukin 28B (IL-28B) (Rao et al. 2012; Thomas et al. 2009) and interleukin 12 (IL-12) genes (Hegazy et al. 2008), human leukocyte antigen (HLA) class I and II alleles (Ocal et al. 2014; Tamori and Kawada 2013; Harris et al. 2008), and strong and sustained HCV multi-specific immune response elicited by $CD4^+$ and $CD8^+$ T cells (Seaberg et al. 2015; Amini and Poustchi 2012).

3.1 Virus-Related Factors

3.1.1 HCV Genotype

The role of the HCV genotype has been mainly demonstrated concerning the efficacy of interferon (IFN) therapies. Despite the fact that therapy for individuals infected with genotype 1 lasts for 48 weeks, its effectiveness reaches 40–50% compared with more than 75% therapeutic success in case of infection with genotype 2 and 3 wherein therapy last for only 24 weeks (Manns et al. 2006). There is no consensus, however, whether the HCV genotype may affect the clinical course of infection or frequency of spontaneous virus clearance. Although some studies suggest genotype 1 be associated with a more severe disease, most authors have found little or no influence of genotype on disease progression. The discrepant findings may stem from biased study designs caused by a failure to control

various important confounding factors, such as the duration of infection, the age, or the disease severity (Harris et al. 2007). There are also contradictory findings about spontaneous viral elimination. Some authors have suggested that infection with HCV genotype 1 is less likely cleared spontaneously compared with other genotypes, while others have not confirmed this association (Grebely et al. 2014; Lehmann et al. 2004; Amoroso et al. 1998; Bruno et al. 1997).

3.1.2 HCV Genetic Heterogeneity

An important feature of HCV is an extraordinary degree of genetic variability. Both the lack of proofreading mechanisms of RNA-dependent RNA polymerase and high HCV replication rate result in the production of mutations during viral replication (Erickson et al. 2001). The degree of diversity varies by more than 30% across the entire genome between the genotypes, 20% between subtypes, and up to 10% within a subtype. HCV circulates in infected individuals as a group of closely related but heterogeneous sequences termed quasispecies (Chen and Wang 2005). The viral genetic variability is not evenly distributed through the entire genome, the highest variable regions include HVR1, HVR2, and HVR3 of the envelope E2 protein (Caraballo Cortes et al. 2013).

The distribution of HCV quasispecies may have important biological consequences. Genetic heterogeneity induced by the immune pressure is considered the primary force driving HCV quasispecies evolution *in vivo*, enabling the virus to escape from humoral and cellular immune responses and to establish chronic infection (Sullivan et al. 2007). During infection course, the highest level of selective pressure occurs in the acute phase and decreases as the infection continues. Sequencing studies suggest that HCV escape mutants are selected at the population level in the context of the prevailing HLA haplotypes. Upon transmission to individuals that do not share identical HLA alleles, HCV spontaneously reverts to its original sequence which is indirect evidence of selection pressure exerted by HLA-restricted $CD8^+$ T cells (Rehermann 2009). A lower level of viral genetic diversity in subjects who cleared viremia *vs.* those who established a chronic infection has been reported (Herring et al. 2005). Progression to chronicity has been related to selective pressure on the HVR1 region of HCV, leading to a rapid evolution of quasispecies. This process results in virus persistence despite the presence of immune response (Fierro et al. 2014).

When the selection mechanisms predominate over genetic drift of the virus, a bottleneck effect can occur. It is defined as a marked reduction in the genetic diversity as a result of elimination of a significant proportion of viral variants (Bull et al. 2011). The bottleneck effect is observed during the ongoing infection and at the time of inter-host transmission (Di Lello et al. 2015). It is responsible for a lower viral genomic diversity in recipients soon after infection when compared with the respective donors even when transmission is caused by a large inoculum (e.g., blood transfusion) (D'Arienzo et al. 2013; Wang et al. 2010). During the initial phase of infection, HCV replicates at a high rate doubling its population every day and generates a highly heterogeneous viral population (Allain et al. 2000). With the development of the acute phase, the fittest variants evolve toward genetic diversification as a result of the immune system pressure. With time of infection, the environment becomes more stable which drives the HCV population toward homogeneity (Di Lello et al. 2015). The subsequent bottleneck effect is responsible for a decline in viral diversity and a decrease in the effective population size observed in subjects who subsequently develop chronic infection (Bull et al. 2011). Moreover, the longer the time of infection, the slower is the viral replication. The HCV evolution is assumed slower due to a relative immune control imposed by the host (Allain et al. 2000).

3.1.3 HCV Proteins

A high rate of HCV persistence suggests the existence of viral inhibitory mechanisms affecting the host immune response. Some HCV proteins are able to attenuate the IFN-mediated

response at multiple levels. A key player is the NS3/4A protein which contains serine protease and RNA helicase activities required for the replication and generation of mature viral proteins. The NS3/4A protein suppresses the host antiviral immune system by cleaving the Toll/interleukin-1 receptor/resistance protein (TIR) domain containing adapter-inducing interferon-β (TRIF), mitochondrial antiviral signaling protein (MAVS), also known as IPS-1, and blocking the toll-like receptor 3 (TLR-3) and retinoic acid-inducible gene I (RIG-I) involved in the production of type I IFNs, such as IFN-α and IFN-β. The induction of IFNs is regulated by the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) that is activated by a signaling cascade generated during the replication of viral RNA. The NS3/A4 protein also suppresses the signaling pathway of activation of NF-κB and in consequence IFN-β production (Li et al. 2005a, b).

Another protein that is liable to have an immunomodulatory function is the HCV core (C) protein. *In vitro* studies indicate that C protein binds to the cytoplasmic domain of members of the tumor necrosis factor-α (TNF-α) receptor superfamily affecting sensitivity of TNF-α mediated lysis of cells expressing this protein. This mechanism may explain the maintaining of virus presence despite the infiltration of liver tissue by virus-specific $CD8^+$ cytotoxic T cells (CTLs). A study of Large et al. (1999) suggests that C protein may be the first viral gene product in the infected cells, suppressing the host immune response at an early stage of infection. Moreover, expression of HCV core protein during infection could explain a low number of CTLs observed in chronically infected individuals and may play a crucial role in maintaining the HCV infection.

The receptor-mediated apoptosis plays an essential role in the HCV-associated liver injury. Some observations indicate the presence of an interaction between C protein and cellular apoptotic pathways, accelerating cell death (Berg et al. 2009; Honda et al. 2000). The C protein interacts with the death domain of Fas-associated protein, enhancing the apoptosis mediated by the latter protein (Bantel and Schulze-Osthoff 2003).

3.2 Host-Related Factors

Among factors associated with spontaneous HCV clearance, female gender and young age are often mentioned (Kong et al. 2014). Seaberg et al. (2015) have demonstrated in an observational cohort study of men who have sex with men that age of 30 years or less is frequently associated with spontaneous HCV clearance compared with men over 30 years of age. Other studies indicate that virus elimination occurs more often in non-blacks (Thomas et al. 2000).

3.2.1 Interleukin-28B (IL-28B)

The IL-28B gene polymorphism has been identified as the strongest independent factor related to spontaneous virus clearance in acute hepatitis C. Since the first studies published in 2009 describing relevance of this marker, many other authors have confirmed the importance of IL-28B (Rauch et al. 2010; Tillmann et al. 2010). IL-28B (IFN-λ3) is a member of type III IFN family that includes IFN-λ1, IFN-λ2, and IFN-λ3. IL-28B's effect is comparable to that of IFN-α and IFN-β, but it is believed that IL-28B binds to a different receptor. Consequently, it upregulates a set of IFN-stimulated genes, promoting an antiviral state in responsive cells (Amini and Poustchi 2012). Three IL-28B genotypes are distinguished: CC, CT, and TT, and individuals with the CC genotype have a stronger immune response to HCV than those with the non-CC genotypes. That makes patients homozygous for CC more likely to spontaneously clear the virus within months of infection. There are ethnic differences in the frequency of specific IL-28 genotypes and the greatest probability of having the beneficial CC genotype display individuals from the Eastern and South-Eastern Asia, while the population of Africans have the lowest CC allele frequency (Table 1) (Stattermayer et al. 2014).

Table 1 Prevalence of IL-28B genotype depending on race, according to Thomas et al. (2009)

Ancestry	Probability of having IL-28B CC genotype (%)
East Asian	90–100
South Asian	65–98
European	53–86
African	23–55

The IL-28B genotype plays a role not only in the frequency of spontaneous virus clearance but also in the effectiveness of the antiviral therapy. A number of studies have shown that IL-28B is a useful predictive factor of response to treatment with pegylated interferon and ribavirin (peg-IFN/RBV). Patients with the CC genotype display a decrease in viral load at the end of the fourth week of therapy, which identifies good responders and a low risk of selection of resistant HCV variants. At the same time, lower importance of this genotype has been shown in the evaluation of treatment effectiveness of new therapeutic regimens, including direct-acting antiviral agents (Rau et al. 2012).

3.2.2 Interleukin-12 (IL-12)

IL-12 has been identified as one of the key cytokines involved in the regulation of primary immune responses. It operates as a regulator of expression of other cytokines such as IFN-γ or TNF-α, enhances the cytotoxic activity of natural killer cells (NK cells), and inhibits IL- 10 production. IL-12 is a T cell-stimulating factor triggering growth and activity of T cells by differentiation of naive T cells into Th1 cells. By this immunomodulatory activity, it promotes type 1 cellular response that boosts the antiviral state.

IL-12 is a unique heterodimeric cytokine composed of two unrelated disulfide-linked subunits – p35 and p40. The subunits are encoded by two separate genes mapping to different chromosomes: p35 (IL-12A) to 3p12-3q13.2 and p40 (IL-12B) to 5q31-q33. The *IL-12B* gene is polymorphic with a functional single nucleotide polymorphism (SNP) A/C of the 3′ untranslated region (3'UTR) at position 1188. The variant C allele of the polymorphism has been associated with enhanced IL-12 production (Hegazy et al. 2008; Latsi et al. 2003). As the susceptibility and elimination of HCV depends on the integrated activities of the immune system and cytokine orchestration, IL-12 plays a crucial role in this mechanism. Studies have demonstrated that the level of IL-12 is lower in patients with viremic, chronic hepatitis C than in those who spontaneously clear the virus. This phenomenon correlates with frequency of C allele responsible for a high IL-12 production. In particular, the homozygous CC genotype is associated with an apparent resistance to HCV infection manifested by the absence of HCV RNA despite a long history of high risk behaviors, including drug injections with needles shared with HCV-infected patients (Hegazy et al. 2008).

3.2.3 HLA Alleles

As a result of the evolutionary adaptation to changing nature of infectious antigens, HLA loci demonstrate an extraordinary rate of diversity. The *HLA* genes in humans correspond to the major histocompatibility complex (MHC) genes that also play a vital role in the immune response to HCV. Although early studies have revealed an association between genetic diversity of HLA class I and II genes and natural history of HCV infection, the exact mechanism is poorly understood.

HLA class I molecules present native and foreign peptides to cytotoxic T cells that are responsible for controlling viral replication during the early stage of infection. A number of studies have confirmed the association between different alleles of these molecules and the development of chronic HCV infection or virus

elimination (Ocal et al. 2014; Chuang et al. 2007; Thio et al. 2002). Although there are some contradictions caused by variability in the study designs, a consensus exists that certain HLA class I alleles correlate with the antiviral protection. The strongest association has been observed between virus clearance and HLA-B27 and HLA-B57 (Amini and Poustchi 2012).

Special attention has been focused on the HLA class II molecules that are responsible for the binding of exogenously processed antigenic peptides and presenting them to $CD4^+$ Th cells. Class II molecules act as the distinctive pockets in the peptide-binding region, formed by both alpha and beta chains that interact with specific fragments of antigenic peptides, influencing the nature and specificity of the antigen-specific $CD4^+$ T lymphocyte response. In HCV infection, HLA class II genotypes DQB1*03 and DRB1*11 have been frequently associated with the self-limited infections. It is assumed that these alleles may present viral epitopes more effectively than the others do, resulting in a dynamic response to HCV by $CD4^+$ Th cells (Harris et al. 2008).

3.2.4 $CD8^+$ and $CD4^+$ T Lymphocytes Response

$CD8^+$ T cells are known to be the key effector cells that control viral infections. They migrate to infected tissues and mediate virus clearance by cytolytic activity and cytokine secretion. The importance of cytolytic function in HCV infection is suggested by the fact that $CD8^+$ T cell responses coincide not only with a decrease in HCV RNA level but also with the peak of alanine aminotransferase in the blood serum. A crucial role of $CD8^+$ T cells in HCV infection is also supported by the immunogenetic studies that show and association between the specific HLA class I allotypes: HLA-B27, HLA-B57 and clinical outcome of HCV infection (Sung et al. 2014). In acute HCV infection, virus-specific T cell response is remarkably delayed despite the early induction of type I and type III IFN responses. The HCV-specific $CD8^+$ T cells cannot be detected in blood and liver until 8–12 weeks after onset of infection. The mechanism underlying the delayed induction of T cells remains unknown (Shin et al. 2016). A vigorous $CD8^+$ T cell response against multiple epitopes with a robust IFN-γ production by HCV-specific $CD8^+$ T cells correlates with a spontaneous resolution of acute HCV infection. In contrast, chronic evolution of acute HCV infection is associated with weak and transient responses of HCV-specific $CD8^+$ T cells that show a lower ability to proliferate and produce IFN-γ (Sung et al. 2014; Lauer 2013). A functional impairment of HCV-specific $CD8^+$ cells has been reported in chronic HCV infection. This phenomenon may be due to a primary T cell failure or impaired antigen presentation. The other possible mechanisms responsible for the failure of immune responses against HCV are the following: direct effect of viral proteins on T cells, lack of memory T cells generation, suppression by regulatory T (Treg) cells, and impaired T cell maturation (Valva et al. 2014; Penna et al. 2007).

$CD4^+$ Th lymphocytes, which display direct antiviral effects, are capable of supporting the cellular mechanisms by producing IFN-γ and of antibody production by secreting IL-2, IL-4, IL-5, and IL-6. A positive association has been found between the $CD4^+$ Th cell-mediated response and spontaneous elimination of HCV in individuals with acute infection (Lauer 2013). Gerlach et al. (1999) have shown that broadly directed and vigorous $CD4^+$ T cell proliferative responses in the first 6 months after infection are crucial for the resolving of HCV infection, regardless of the level of viral replication. Moreover, in patients developing only a transient virus-specific T cell response, who are able to eliminate HCV RNA from the serum, a reappearance of HCV RNA is observed after the loss of a specific response.

A weak and directed against few viral epitopes cellular response has been detected in subjects developing a chronic form of HCV infection (Gerlach et al. 1999; Higashi et al. 1996). In these patients, the percentage of HCV-specific $CD4^+$ T cells in the peripheral blood is usually very low or even undetectable, and the cells are defective in their ability of

proliferation and cytokine secretion (Kazmierczak et al. 2016). Nonetheless, in chronic HCV carriers, the presence of $CD4^+$ T cells reactive to the core or non-structural antigens has been demonstrated in the liver. These findings suggest that specific responses of HCV-specific $CD4^+$ T cells may be restricted to the location that is the primary site of inflammation.

4 Spontaneous HCV Clearance During Chronic Infection

The diagnosis of chronic HCV infection can be determined by the presence of HCV RNA in the patient serum for more than 6 months (Seeff 2002). A chronic HCV infection lasting many years may result in progressive liver fibrosis and cirrhosis, and in primary hepatocellular carcinoma. Since most chronic HCV carriers are asymptomatic, spontaneous elimination of HCV viremia occurs unnoticeably. Studies reveal that a spontaneous clearance of HCV infection in the chronic phase is rare and its mechanisms are not well understood (Heim et al. 2016). Depending on the population studied and the survey conditions, various rates of spontaneous virus clearance during chronic infection have been demonstrated. In a recent study performed in a large Scottish cohort, clearance rate has been 0.36/100 person-years (Bulteel et al. 2016), while another study employing a significant percentage of Alaskan population has given a score of 1.15/100 person-years (Scott et al. 2006). The discrepancy reported in the spontaneous HCV elimination may be due to genetic differences and other factors. For instance, spontaneous clearance in chronic HCV infection has been reported after liver transplantation, withdrawal of immunosuppressive medications, following superinfection with hepatitis B virus (HBV) or hepatitis delta virus (HDV), and after the initiation of antiretroviral therapy in subjects co-infected with HIV/HCV (Bulteel et al. 2016). In most cases of co-infection, virus elimination is achieved after the immune system reconstitution as a result of antiretroviral treatment. Importantly, individuals in whom the virus elimination occurs harbor the IL-28B CC genotype (Stenkvist et al. 2014; Vispo et al. 2014).

5 Conclusions

Although for almost 30 years that elapsed from the identification of HCV much progress has been made in the understanding of the pathogenesis of the infection and antiviral treatment, some aspects still remain unexplained. The host immune response plays a principal role in HCV spontaneous clearance. Nonetheless, the virus uses a vast array of mechanisms to evade the adaptive and innate immune defense, e.g., by generating the escape mutants. Clinical studies on the spontaneous elimination of HCV infection are problematic due to difficulties in the recruitment of a large cohort of acute patients and the need for an accurate follow-up to ensure the virus elimination, with all the inherent differences arising from the patient ethnicity and experimental conditions. Consequently, studies on certain aspects of the spontaneous elimination of HCV are inconsistent. Further research is needed to get a detailed insight into the mechanisms of virus eradication.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Clinical Manifestations of Huge Diaphragmatic Hernias

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Abstract

Translocation of abdominal organs into the thoracic cavity may cause dyspnea, heart disorders, and gastric symptoms. Diaphragmatic hernias can cause diagnostic difficulties, since both clinical and radiological symptoms might imitate different disorders. In these cases computed tomography of the chest is the method of choice. The aim of this study was to assess clinical manifestations, risk factors, and prognosis in patients with huge diaphragmatic hernias with displacement of abdominal organs into the thorax, depending on the action taken. We carried out a retrospective study using data of patients hospitalized in the years 2012–2016. Ten patients were qualified for the study (8 women and 2 men). The mean age of the subjects was 86.5 ± 10.5 years. Thirty percent of the hernias were post-traumatic. All of the patients reported cardiovascular or respiratory symptoms. Upper gastrointestinal symptoms occurred in half of the patients. Twenty percent of patients underwent surgery with a positive outcome, while 30% of patients, who were not qualified for surgery due to numerous co-morbidities, died. The main risk factors predisposing to the occurrence of large diaphragmatic hernias were the following: old age, female gender, and thoracic cage deformities.

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Keywords
Dyspnea • Esophageal hernia • Gastric symptoms • Retrosternal hernia • Respiratory failure • Risk factors • Stomach

1 Introduction

The thoracic cage is a confined space dedicated to the protection of vital organs needed to survive, such as the heart, lungs, and large blood vessels. At times, organs from other parts of the body move into the chest. A typical example of such a translocation is a retrosternal goitre (Mercante et al. 2011). Another example are diaphragmatic hernias (Eren and Ciriş 2005). At the same time, the diaphragm plays the role of a muscular dome separating the thorax form the abdomen and the role of the main respiratory muscle. Natural orifices in the diaphragm and regions with a thinner muscle layer are areas with decreased resistance, which can become the gateways for diaphragmatic hernias. The movement of organs from the abdomen into the thoracic cavity can manifest with respiratory, circulatory, and gastrointestinal symptoms of varying intensity.

Acquired diaphragmatic hernias can be divided into idiopathic, iatrogenic, and post-traumatic. Cases of iatrogenic hernias due to liver transplants (Cortes et al. 2014) and after the use of positive-pressure non-invasive ventilation have been described (Tone et al. 2014). Post-traumatic diaphragmatic defects make up 10% of all diaphragmatic disorders and over 90% of them are located on the left side (Nursal et al. 2001). Blunt abdominal or thoracic trauma can cause asymptomatic diaphragmatic hernias or ones with very few symptoms (Kaur et al. 2015). Occasionally, diaphragmatic hernias are discovered during pregnancy when, due to an increase in abdominal pressure, symptoms appear as a cause of an enlarging diaphragmatic hernia (Schwentner et al. 2011). Esophageal hernias are fairly common but rarely attain large sizes. In the elderly, they can cause significant clinical symptoms considerably decreasing their quality of life and therefore may need surgical intervention (Le Page et al. 2015).

The aim of the present work was to assess clinical symptoms, risk factors, and prognosis depending on the measures undertaken in patients with extensive diaphragmatic hernias with displacement of abdominal organs into the thoracic cavity.

2 Methods

The institutional Ethics Committee waived the obligatory permission to conduct the study in accordance with current regulations stating that retrospective studies of anonymous clinical cases, presented by practicing physicians, do not need to have ethical approval. In this report we presented a series of 10 cases of large diaphragmatic hernias which were diagnosed in the years 2012–2016 in the Czerniakowski Hospital in Warsaw, Poland. The group investigated consisted of 8 women and 2 men and their ages ranged from 67 to 96 years, with the mean age of 86.5 ± 10.5 years (Table 1). The patients, at the time of admission, did not have a diagnosis of diaphragmatic hernia established, but were hospitalised due to various other reasons. The diagnosis was based on computed tomography of the chest together with an evaluation of the upper abdominal areas.

3 Results

All of the patients reported cardiovascular, respiratory, or gastric symptoms and there were no cases of asymptomatic huge diaphragmatic hernias detected accidentally using imaging studies (Table 1). Dyspnea was present in 80% of patients; 30% of whom had dyspnea at rest and

Table 1 Clinical characteristics of patients

Patients	1	2	3	4	5	6	7	8	9	10
Sex (M male/F female)	M	F	F	F	F	M	F	F	F	F
Age (years)	70	67	95	94	92	96	87	82	95	87
Symptoms (Yes +/No –)	+	+	+	+	+	+	+	+	+	+
Dyspnea (Yes +/No –)	+	+	+	+	+	–	–	+	+	+
Pain of thorax (Yes +/No –)	+	+	–	–	–	–	+	+	+	+
Cough (Yes +/No –)	–	–	–	–	+	–	–	–	–	–
Tachypnea (per min)	26	>30	30	<20	22	<20	26	<20	22	14
Tachycardia (per min)	98	160	115	70	90	50	88	84	108	69
Arrythmia (Yes +/No –)	–	+	+	–	+	+	–	–	+	–
Auscultation sounds (Yes +/No –)	–	+	–	–	+	+	+	–	+	+
SaO_2 (%)	94	87	88	98	89	–	86	–	100	96
Heart failure (Yes +/No –)	+	+	+	+	+	+	+	+	+	–
Nausea (Yes +/No –)	+	+	–	+	+	–	+	+	–	–
Vomiting (Yes +/No –)	+	+	–	–	–	–	–	+	–	–
Abdominal pain (Yes +/No –)	+	–	–	+	–	–	–	+	–	–
Abdominal tenderness (Yes +/No –)	+	–	–	+	–	–	–	+	–	–
GERD symptoms (Yes +/No –)	+	+	–	+	+	–	–	+	–	+

SaO_2 arterial oxygen saturation, *GERD* gastroesophageal reflux disease

acute dyspnea was found in 10% of patients. Cough occurred very rarely, only in 10% of patients, and it was productive with a purulent secretion, which could be due to a coexisting infection. Sixty percent of patients declared chest pain. In some of the cases it occurred after thoracic trauma and was located in the area of impact. In the remaining cases it was retrosternal, but not characteristic of anginal pain, since it was associated with food intake. Tachypnea (>20 breaths/min) was found in 60% of patients, tachycardia (>90 beats/min) in 40% and bradycardia (<60 beats/min) in 10%. In 60% of patients, abnormal breath sounds were heard over the lung fields. Most often these were symptoms of congestion due to coexisting heart failure. In one patient, who had a Morgagni hernia with translocation of the intestines into the thorax, peristalsis could be heard on chest auscultation. Arrhythmias were found in 50% of patients. The ECG showed permanent atrial fibrillation (AF) in 2 patients and paroxysmal AF in one. In one patient, numerous extrasystolic beats were found, while in another patient bradycardia was observed. Respiratory failure (SaO_2 <92%) was noted in 40% of patients and heart failure in 90% of patients. Sixty percent of patients reported gastric symptoms (Table 1). Nausea and vomiting were predominantly described, occurring in 60% of patients.

Laboratory tests showed hyponatremia (Na <136 mmol/L) in 50% of patients, while in 20% the sodium level was below 130 mmol/L (Table 2). Hypokalemia was rarely observed (10% of patients). Normocytic anemia was noted in 60% of patients, but was mild in most cases. Renal injury, due to dehydration caused by nausea, vomiting, and decreased fluid intake, was observed in 40% of patients. In 50% of patients an increase in C-reactive protein was noted. D-dimer levels were checked in 80% of patients and they were elevated in all of those cases (531–8166 ng/mL). Biochemical features of liver damage were not observed in any of the patients. Abdominal ultrasound examination did not contribute any significant information in the tested group. Gastroscopy, which was performed in 20% of patients, confirmed large esophageal hernias. In all of the cases, since the group in question consisted of elderly people, multiple coexisting chronic disorders were found (2–6 disorders). The most commonly diagnosed were cardiovascular disorders, such as hypertension, ischemic heart disease, myocardial infarction, stroke, or chronic thromboembolic disorder, and lung disorders, including chronic obstructive

Table 2 Results of biochemical and clinical examinations in patients with diaphragmatic hernias

Patients	1	2	3	4	5	6	7	8	9	10
Na^+ (mmol/L)	137	135	124	126	141	141	131	143	142	131
K^+ (mmol/L)	3.6	3.0	4.4	4.4	4.0	4.2	5.0	4.7	4.8	4.6
Hb (g/dL)	12.4	10.1	13.1	13.4	12.0	11.7	11.7	10.3	13.8	11.9
CRP (mg/dL)	14.0	22.6	21.0	NS	6.1	0.4	6.6	0.9	2.4	<0.4
D-dimer (ng/mL)	855	8166	531	NS	1391	1112	2895	NS	2246	2895
GFR (mL/min/1.73 m^2)	>60	>60	>60	20.0	>60	50.8	45.6	68	30.5	>60
ALT (UI/mL)	18	10	12	19	20	18	28	9	11	17
Liver USG	N	NS	N	KC	KS	NS	N	NS	N	NS
Co-morbidities (n)	6	2	5	5	5	4	2	5	5	2

Hb hemoglobin, *CRP* C-reactive protein, *GFR* glomerular filtration rate, *ALT* alanine aminotransferase, *USG* ultrasonography, *N* normal picture, *NS* no study, *KC* kidney cysts, *KS* kidney stone

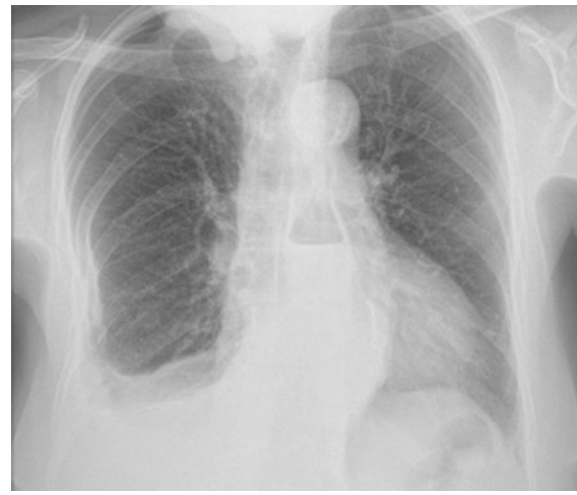

Fig. 1 Posterior–anterior chest X-ray. An image of a large sliding hernia, with the gastrointestinal tract visible in the midline

pulmonary disease, emphysema, and pneumothorax. Prostate and skin cancer were diagnosed in 20% of patients. There were individual cases of depression, diabetes, ulcerative colitis, bile duct stricture post gallstone removal, nodular goitre and arthritis.

In some of the cases, chest X-rays were enough to conclusively make the right diagnosis (Fig. 1). In as many as 70% of cases, despite the marked sizes of the diaphragmatic hernias, the radiologist commenting on the chest X-ray did not suggest such a diagnosis. The proposed diagnoses included: pneumothorax, inflammatory changes, tumor with necrosis, thick-walled cavity with emphysema, and a solid lung consolidation connected to the hilum. In 20% of the post-traumatic cases, a pleural effusion was noted. In cases in which the radiological picture was unclear (Fig. 2), chest CT imaging was used to clear any uncertainties (Fig. 3). The hernias reached sizes from a couple to around a dozen centimetres; the largest measured 10 × 21 cm (Fig. 4). Severe kyphoscoliosis was very frequently noted, sometimes together with fractures of thoracic vertebrae and in one case together with fractured ribs (Fig. 5). A severe thoracic deformity was not observed only in the case of a post-traumatic Morgagni type of hernia.

Large esophageal hernias, with translocation of the stomach into the thoracic cage, dominated in the collected data (90%). Only in one case, there was a post-traumatic Morgagni type hernia

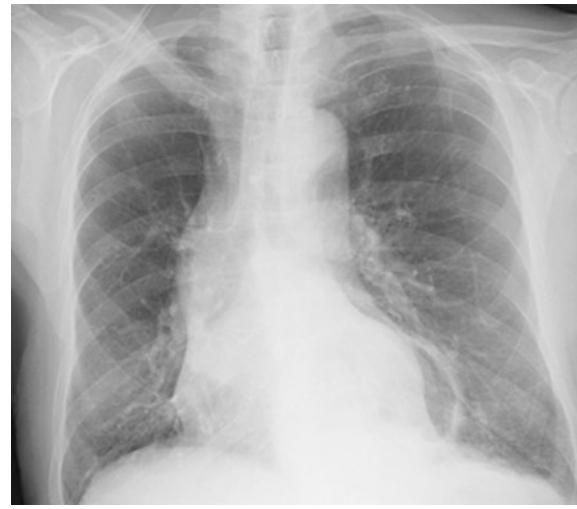

Fig. 2 Posterior-anterior chest X-ray. An unclear shadow was described around the left side of the heart

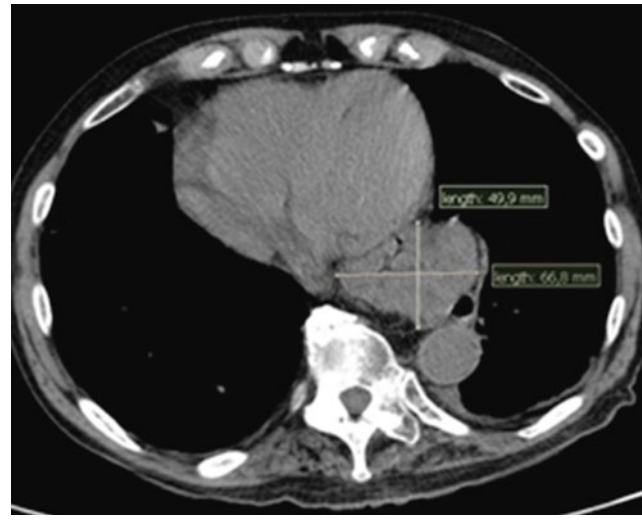

Fig. 3 High resolution computed tomography (HRCT), with no contrast, of the patient whose chest X-ray is shown in Fig. 2. The undefined chest shadow was now diagnosed as a large hiatns hernia with stomach displacement into the chest

noted (Fig. 6). In 30% of patients, hernias were due to trauma, with coexisting fractures of ribs, spine, and pelvis. Only in 20% of patients, surgery was performed with good long-term effects; lasting for a couple of years (Table 3). However, over half of the patients did not qualify for surgery due mainly to a very advanced age; over 90 years. In one individual, serious coexisting disorders disqualified for surgery and in another case younger patient refused to give consent for the proposed surgical management. In 30% of patients, enormous diaphragmatic hernias were one of the main reasons why conservative treatment failed and the patients died. Non-surgical treatment was largely based on adequate dietary recommendations which, in a couple of cases, led to a decrease in symptoms (Table 3). Nevertheless, in most of the cases, the patient surveillance was short and based on the duration of the hospital stay which was a couple of weeks long.

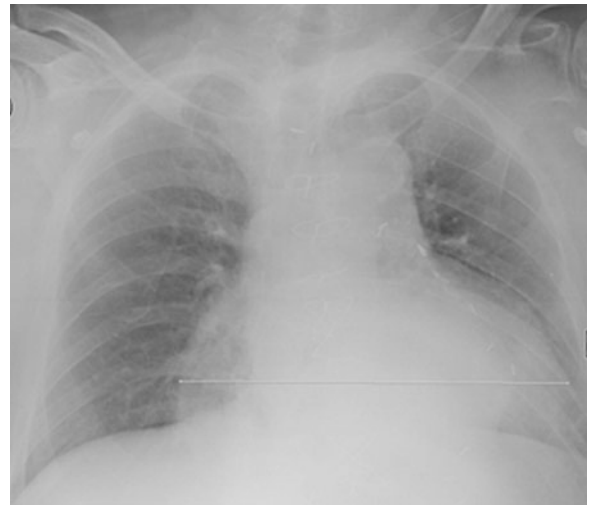

Fig. 4 Posterior–anterior chest X-ray. A large diaphragmatic hernia is visible at the base of the lung with a diameter of 21 cm

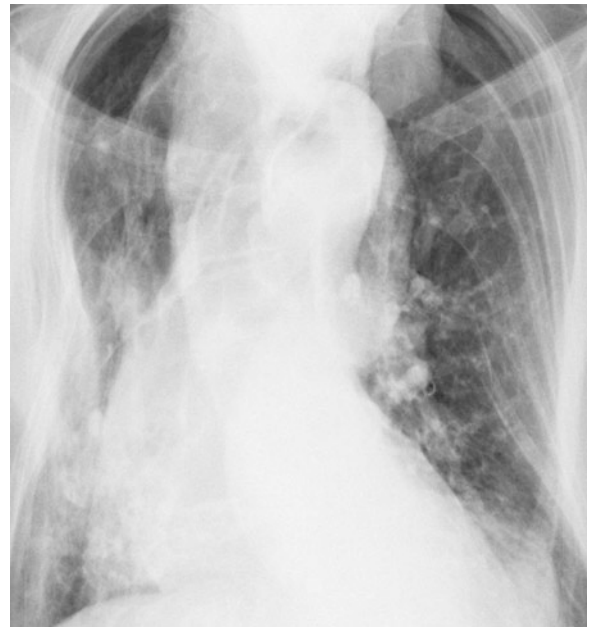

Fig. 5 Posterior–anterior chest X-ray. Significant distortion of the chest caused by a large hiatal hernia with displacement of the entire stomach into the chest

4 Discussion

Esophageal sliding hernias are not a rare finding. Higher frequencies of hiatal hernias have been reported in Western populations, for example, 16.6% in Norway (Berstad et al. 1986), 22% in the USA (Wright and Hurwitz 1979), and 14.5% in Sweden (Cronstedt et al. 1978). In this paper, a collection of huge hernias was compiled, in which the whole stomach or most of it, translocated into the thoracic cage. In the presented cases of large diaphragmatic hernias, the variability of the course of illness is evident. The character and combinations of clinical symptoms depended on the cause and time in which it developed. An acute, quickly progressing course of illness, leading to the

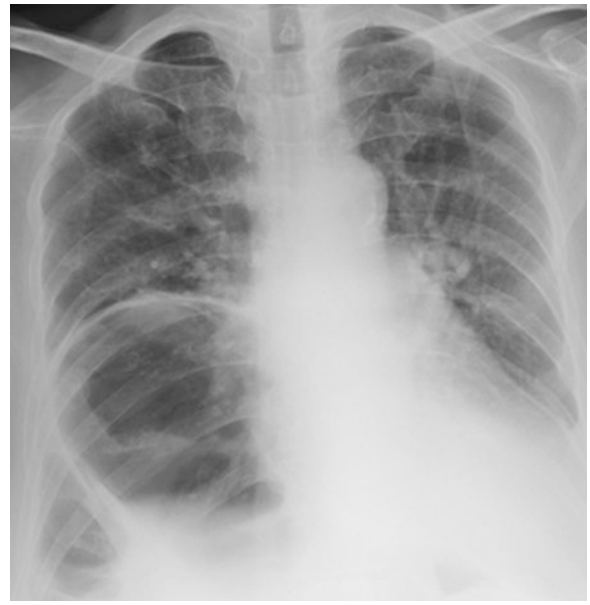

Fig. 6 Posterior–anterior chest X-ray. Post-traumatic Morgagni type hernia with displacement of the stomach and small intestine into the chest

appearance of dyspnea at rest and symptoms of respiratory and cardiovascular failure, was observed in post-traumatic cases. In the remaining instances, large diaphragmatic hernias were diagnosed accidentally, based on abnormalities in the radiological imaging of the thoracic cage, although patients always reported some kind of respiratory, cardiovascular, or gastric symptoms. Symptoms of cardiovascular failure were present in 90% of patients, but they were rather connected with coexisting disorders and with older age. Pulmonary symptoms reported by 80% of patients always accompanied an acute course of the disorder or when there were multiple coexisting conditions; for instance, heart failure. Heart rhythm abnormalities were not such a common condition. However, gastric symptoms were present in over half of the cases, with 60% of patients reporting symptoms of gastroesophageal reflux.

Laboratory tests did not add any diagnostically useful information and their only role was the evaluation of coexisting illnesses. The fact that specialist radiologists often incorrectly interpreted radiological changes and despite the large sizes, did not suggest the presence of diaphragmatic hernias was somehow distressing. It was not until the chest CT was done that the diagnosis was made, even though the diagnosis could have been suspected based on the chest X-ray. Likewise, ultrasonography, which could be useful for the diaphragmatic hernia diagnosis, did not appear much of help.

The patients examined in this study can be divided into two subgroups. The first subgroup consisted of 30% of patients, in whom an acute post-traumatic hernia occurred, with symptoms visibly intensifying, sometimes affecting various organs. This group was characterized by a significantly younger age (74.4 $\pm$ 12.0 years). The course of illness was very dramatic in a 67-year-old woman. She had a post-traumatic fracture of the proximal left femur, post-surgical cardiopulmonary failure, and persistent vomiting. That was due to a Morgagni type retrosternal diaphragmatic hernia, with translocation of the stomach and sections of the small intestine into the thoracic cage. The patient had a prompt surgical intervention, with good long-term results achieved. On the other hand, a 70-year-old man suffered from fractures of vertebrae Th10 and Th11, and the arch C7, after a motor vehicle accident. He presented with a bilateral pleural effusion and translocation of the

Table 3 Hernia characteristics and treatment effects

Patients	1	2	3	4	5	6	7	8	9	10
Post-traumatic hernia	+	+	−	−	−	−	+	−	−	−
Hiatal hernia	+	−	+	+	+	+	+	+	+	+
Chest X–ray conclusive	+	−	+	−	−	−	−	−	−	+
Hernia dimension (cm)	9.9 × 20.9	6.0 × 14.0	9.1 × 12.9	9.6 × 9.7	6.1 × 4.3	5.0 × 6.3	8.1 × 8.3	5.5 × 6.8	8.5 × 3.3	5.0 × 3.5
Kyphoscoliosis/fracture	+	−	+	+	+	+	+	+	+	+
Surgical treatment	NQ	+	NQ	NQ	NQ	NQ	NC	+	NQ	NQ
Improvement after treatment	−	+	−	+	+	+	+/−	+	−	+
Death	+	−	+	−	−	−	−	−	+	−
Survival (months)	4	12	1	1	24	1	1	48	1	12

NC No consent, *NQ* Not qualified, Yes +, No −

stomach into the thorax. Despite the diagnosis of a large diaphragmatic hernia, in the 3 months following the accident, surgery was not considered until he was admitted to the hospital with increasing cardiovascular and gastric symptoms. He was disqualified from surgery because of numerous co-morbidities and ultimately died after a couple of months due to malnourishment caused by persistent vomiting and worsening heart failure. A third case was an 87-year-old woman who sustained chest trauma after falling onto a chair. She fractured ribs VI-IX on the right-hand side, and presented with a pleural effusion with symptoms of a large sliding hernia with translocation of the whole fundus of the stomach into the thoracic cage. The patient did not consent to surgery and was discharged after stabilization of her general condition.

In all of the cases outlined above diaphragmatic hernias were diagnosed shortly after the trauma. However, long-term consequences have also been described when, for example, bowel obstruction due to diaphragmatic hernia occurred after 9 months (Bhatti and Dawani 2015). Sometimes large post-traumatic hernias presenting with few symptoms are diagnosed a couple of months, or even years, after the incident (Kumar et al. 2013; Menéndez-Sánchez et al. 2012). Hernia tears occur in 0.8–1.6% of acute blunt abdominal traumas (Troop et al. 1985). Such hernias are uncommon, but very dangerous and have an overall mortality rate of up to 31% (Morgan et al. 2010). Traumatic diaphragmatic hernias are sometimes difficult to identify at an early stage and can consequently result in diagnostic delays. The interval from injury to developing symptoms may range from 2 to 11 years (Lu et al. 2016). Therefore, it is not possible to exclude the possibility that a large diaphragmatic hernia could occur in the distant future after trauma. This indicates a need for a routine radiological assessment of the thoracic cage after trauma to the abdomen or chest. This will allow an early identification of serious post-traumatic consequences that can be missed when the diagnosis is, in the first instance, based on scarce clinical symptoms. The gold standard for diagnosing diaphragmatic tears is an ultrasound examination, which has been estimated to have a sensitivity of 82% (Pfannschmidt et al. 1994). Ultrasonography also diagnoses hiatal hernias (Cakmakci et al. 2014). A much more precise method is CT imaging (Gmachowska et al. 2016). A magnetic resonance imaging (MRI) can also be helpful in such cases, as it visualizes well the soft tissues near the thoracic wall (Dancewicz et al. 2006).

In two of the cases described in the present report, surgical management had very good long-term effects. However, the 30% of patients, in whom surgery could not be performed due to various reasons, died, due in part to large diaphragmatic hernias in each of the cases. Sliding hiatus hernia is often associated with reflux and usually treated medically, but surgery is needed for some patients. Para-esophageal hernia may also be a serious problem with major complications (Blamey 1998). In the case of a large or symptomatic hernia, the only treatment method, with a possibly good long-term outcome, is a swift surgical intervention (Arora et al. 2008; Horton et al. 2008). Surgical treatment is recommended for all the patients with this pathology because of high risk complications such as obstruction, incarceration, strangulation, or perforation. A laparoscopic approach is gaining in importance because it is an effective and safe method of treatment of even large sized hernias (Yavuz et al. 2006). Since this technique is considered to be difficult, surgeons have started using the Da Vinci robot in cases of huge diaphragmatic hernias (Morelli et al. 2015).

The second subgroup consisted of patients in whom the diaphragmatic hernia was not believed to be post-traumatic. What stands out is the older age in this group (an average of 91.6 ± 5.2 years). It seems that the prognosis in older patients is worse, principally due to the coexistence of multiple disorders. One can assume that as a person ages their elastic tissues become more flaccid and so their effectivity as a barrier for individual organs decreases. This can lead to a significant translocation of abdominal organs into the thoracic cavity. An increase in longevity widely observed in highly developed countries causes these changes to become

evident more often. A diagnosis of such large hernias in people over the age of 80 will have a significant influence on their quality of life and a poor long-term prognosis. In this subgroup, there were no Morgagni type hernias that are congenital, although are not rarely diagnosed in adults (Horton et al. 2008). At an older age, the development of diaphragmatic hernias may be related to external factors; e.g., trauma, or internal factors, e.g., obesity, degeneration of connective tissue progressing with age, or surgery. Diaphragmatic hernias specifically in the aged population of patients have not yet been presented in the literature, although age is considered a risk factor of paraesophageal hernias (Collet et al. 2013). The older age and associated co-morbidities cause significant limitations when considering surgical intervention, which therefore might have lethal consequences (Awais and Luketich 2009).

The patients of the present study were women in 80%. Earlier papers have also noted an increased occurrence of large hernias in women. This is confirmed by the articles from Japan that show a predominance of women among patients with diaphragmatic hernias (Kusano et al. 2008; Fujimoto 2004). This was tied to another risk factor that characterizes the group in question, which is significant deformities of the thoracic cage such as kyphosis, kyphoscoliosis, and fractures of ribs or vertebrae. These changes were observed in almost all of the our patients. Epidemiological studies in Japan have found that an increased incidence of kyphosis and osteoporosis in the elderly and female population may lead to the development of hiatal hernias (Fujimoto 2004). Fractures of vertebrae connected with osteoporosis in elderly women also correlate with a frequent occurrence of diaphragmatic hernias and gastroesophageal reflux (Yoshimura et al. 2008). Enlargement of the anterior-posterior diameter of the thoracic cage occurs in the elderly and is associated with the occurrence of hiatal hernias (Masaoka et al. 2012). Among the risk factors of large diaphragmatic hernias mentioned in the literature, obesity has also been indicated (Che et al. 2013), but this concerns a much younger population; an average age of 44 years and a BMI of 43 kg/m^2. However, Japanese studies show that in elderly people the size of hiatal hernias depends rather on kyphosis than obesity. These studies demonstrate wide differences between diaphragmatic hernia risk factors in men and women. In men, obesity plays an important role, while in women age and thoracic deformities are more important (Kusano et al. 2008).

In conclusion, the presented series of cases shows that huge diaphragmatic hernias can cause diverse respiratory, cardiovascular, and gastric symptoms. Factors particularly predisposing to the occurrence of diaphragmatic hernias are older age, female gender, and thoracic cage deformities.

Conflicts Interest The authors declare no conflict of interest in relation to this article.

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The Diagnostics of Human Steroid Hormone Disorders

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Abstract

Disturbances of the steroidogenesis or altered peripheral metabolism of steroids may result in severe clinical manifestations. Therefore, prompt diagnosis and initiation of medical treatment are desirable. The diagnostics of disorders of steroid hormone production, metabolism, and action have been previously based on immunoassay tests. However, in a modern medical laboratory, due to low accuracy of immunoassays, this technique is continuously replaced by chromatographic separation methods coupled to mass spectrometric detection systems. In this review we present current advances in the diagnostics of adrenal gland disorders, focusing on the role of mass spectrometry in prenatal and newborn screening, and in the diagnostics of sexual maturation disorders.

Keywords

Adrenal insufficiency • Diagnostics • Hormonal metabolism • Mass spectrometry • Sexual maturation disorders • Steroid hormones • Steroidogenesis

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1 Introduction

Adrenal steroid hormones are synthesized from cholesterol, a normal constituent of lipid bilayers, in a series of coordinated reactions catalyzed by multiple enzymes. The cascade of transformation of the neutral lipid cholesterol into steroid hormones consists of 11 different bioconversions involving a series of hydroxylation, oxidation, and reduction steps. As a consequence, a vast array of biologically active compounds: mineralocorticoids, glucocorticoids, and sex hormones are formed. The majority of these transformations occur in the adrenal glands, testes, and ovaries. Moreover, steroid hormones can be synthesized within other tissues such as liver, kidney, placenta, brain, and skin. The complexity of the steroid synthesis pathway is shown in Fig. 1.

Adrenal insufficiency is a clinical manifestation of deficient production or activity of glucocorticoids, which can be accompanied with deficiency in mineralocorticoids and adrenal androgens. It is a life-threatening disorder, in pediatric patients resulting mostly from primary adrenal failure, and a prompt diagnosis and management are essential to implement successful treatment. The disorder can result from different forms of congenital adrenal hyperplasia, adrenal salt wasting, global adrenal insufficiency, tumors, or altered peripheral metabolism of adrenocortical hormones (Taylor et al. 2015). Clinical manifestations of primary adrenal insufficiency result from deficiency of all adrenocortical hormones, and the diagnostic investigation can be challenging. The diagnosis of various disorders is based on the measurement of a content of circulating steroid end-products and synthesis intermediaries, present at very low or extremely low concentrations. The low levels and chemical similarity of analogs makes the assessment of endogenous steroids difficult, especially in infants and children as the blood sample volume is limited. The accuracy of steroid assays is a major concern in the clinical evaluation of congenital or acquired adrenal disorders and steroid hormone metabolism.

The measurement of steroid hormones and their precursors/metabolites plays an important role in clinical diagnostics, but despite the rapid development of available techniques, it remains problematic. Quantification of an individual steroid hormone singled out from a spate of structurally similar compounds (Table 1) is extremely challenging. In fact, differentiation between steroid hormones, as well as between their metabolites, often relies on the steric conformation of a molecule or the presence or absence of hydrogens in the molecule (Andrew 2001). The difficulties are associated not only with structural similarity among hormones, but also with existing differences between the steroid profile in the bloodstream and their metabolites in the urine, or the presence of binding proteins inactivating their activity.

Nowadays, mass spectrometry (MS) is considered the best method for the diagnostics of adrenal insufficiency, allowing for a reliable and comprehensive analysis of multiple compounds at the same time. The utility of MS is not limited to the diagnostics of adrenal insufficiency. It is also widely used for the detection of adrenal steroid hormones overproduction. In this review we present current advances in the diagnostics of adrenal gland disorders, focusing on the role of MS in a modern medical laboratory in prenatal and newborn screening, and also in the diagnostics of sexual maturation disorders.

2 Prenatal Diagnostics of Adrenal Disorders

Longitudinal monitoring of pregnancies carrying fetuses with adrenal disorders resulting in defects in the sex development pointed to several biochemical markers, which can be useful for prenatal diagnostics of these inborn errors. Steroid profiling of maternal urine with the use of MS techniques is currently employed for prenatal detection of the Smith Lemli Opitz syndrome (SLOS) and congenital adrenal hyperplasia caused by P450 oxidoreductase deficiency (PORD).

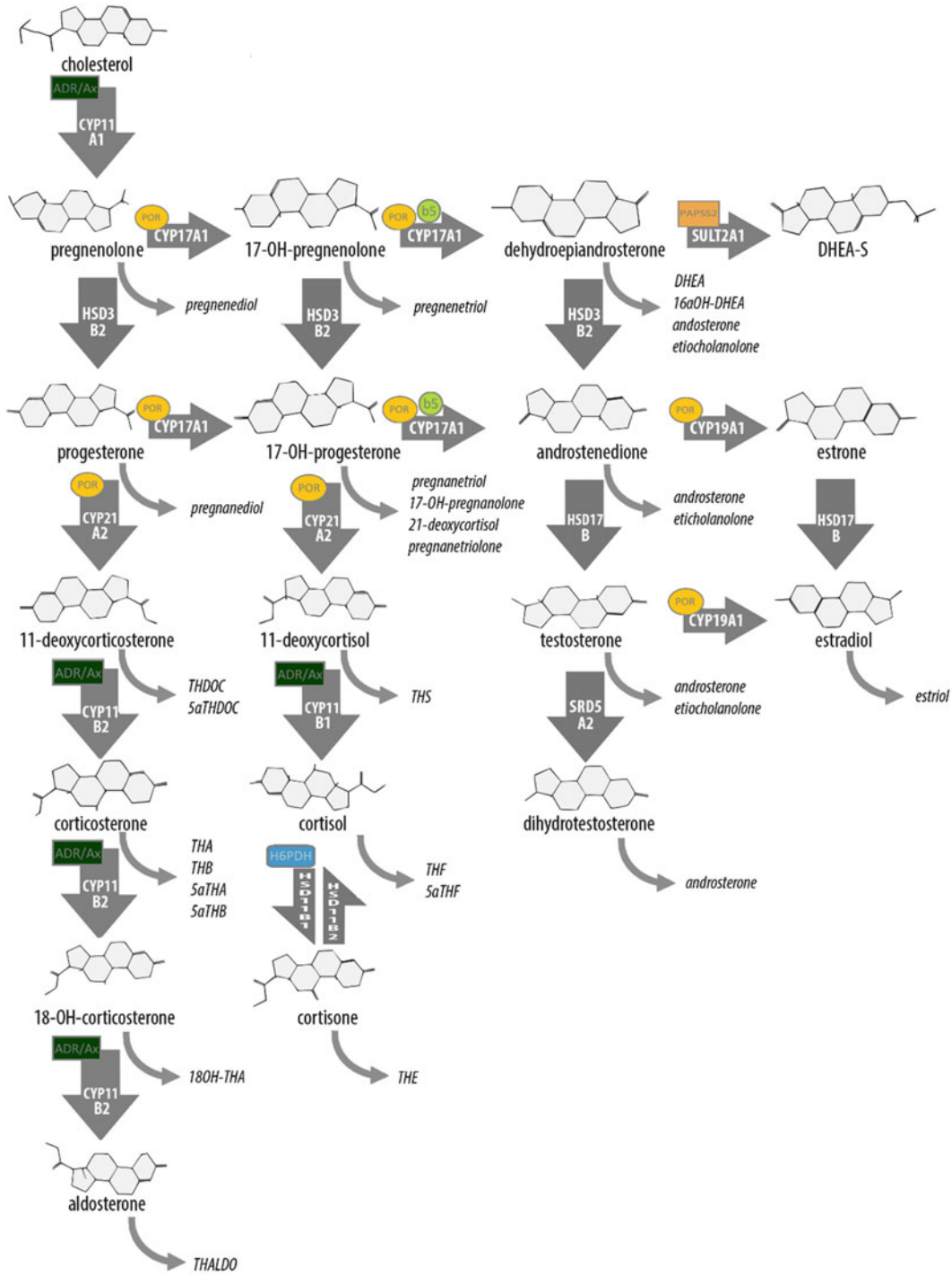

Fig. 1 Synthesis and metabolism of hormonal steroids. *ADR* adrenodoxin reductase, *Adx* adrenodoxin, *POR* P450 oxidoreductase, *b5* cytochrome b5 *SULT2A1* sulfotransferase 2A1, *PAPPS2* 3′-phosphoadenosine-5′-phosphosulfate synthase 2, *CYP19A1* P450 aromatase, *SRD5A2* 5α -reductase type 2, *CYP11B2* aldosterone synthase, *H6PDH* hexose-6-phosphate dehydrogenase, *HSD17B* 17α -hydroxysteroid dehydrogenase, *HSD3B2* 3β-hydroxysteroid dehydrogenase type 2, *CYP17A1* 17-α-hydroxylase, *CYP11B1* 11β – hydroxylase, *THE* tetrahydrocortisone, *THF* tetrahydrocortisol

Table 1 Metabolites of steroid hormones

Abbreviation	Trivial name
AN	Androsterone
ET	Etiocholanolone
11-OAN	11-ketoandrosterone
11-OET	11-ketoetiocholanolone
11-OHAN	11-hydroksy-androsterone
11-OHET	11-hydroksy-etiocholanolone
DHA	Dehydroepiandrosterone
Δ5-and	Androstendiol
5-and	5-androstenediol
16a–OHDHA	16α-hydroxydehydroepiandrosterone
An-3-ol	6α-hydroksyandrostenediol
5-Pt	3β,17α,20α-trihydroxy-5-pregnene
16-OHPN′	16α-hydroksypregnenolone
17-OHPN (5β)	17-hydroksypregnanolone (5β)
17-OHPN (5α)	17-hydroksypregnanolone (5α)
15β,17α-dOHPN	15β,17α-dihydroxypregnanolone
PT	Pregnanetriol
Δ5-Pt	Pregnentriol
Δ5-Pt (16α-oh)	Pregnentriol (16α-OH)
PTN	Pregnanetriolone
PD	Pregnanediol
Δ5-PD	Pregnendiol
E1	Estrone
E2	Estradiol
E3	Estriol
THS	Tetrahydroxycortisol
THDOC	Tetrahydro-11-deoxycorticosterone
THA	Tetrahydro-11-dehydrocorticosterone
THB	Tetrahydrocorticosterone
alloTHB	Allotetrahydrocorticosterone
THAldo	Tetrahydro-aldosterone
THE	Tetrahydrocortisone
THF	Tetrahydrocortisol
alloTHF	Allotetrahydrocortisol
a-CTN	α-cortolone
b-CTN	β-cortolone
b-CT	β-cortol
a-CT	α-cortol
E	Cortisone
F	Cortisol
6α-OHTHE	6α-hydroxytetrahydrocortisone
6β-OHF	6β-hydrocortisol
20α-DHF	20α/β- dihydrocortisol
18-oxo-THF	18-oxo-tetrahydrocortisol
18-OHF	18-hydroxycortisol
18-oxoF	18-oxocortisol
18-OHB	18-hydroxycorticosterone

SLOS is an autosomal recessive disorder caused by insufficient activity of 7-dehydrocholesterol (7-DHC) reductase, which results in a decreased level of cholesterol and increased concentrations of 7-DHC and 8-DHC in body fluids and tissues. Clinical symptoms in affected individuals are highly variable, ranging from minor physical abnormalities along with behavioral and learning problems, to profound intellectual disability and a considerable physical handicap. The incidence of SLOS is estimated at 1:20,000–1:60,000 worldwide. In Europe this syndrome is commonly observed, e.g., the frequency of carrier mutation *DHRC7* is ranging from 1:26 to 1:32 and the disease incidence is 1:2,300–1:3,937 in the Polish population, being one of the most common metabolic diseases. SLOS is characterized by the increased ratios of 7-dehydropregnanetriol/pregnanetriol (7-PT/PT), 8-dehydropregnanetriol/PT (8-PT/PT), 7+8-PT/PT, and dehydroestriol/estriol (DHE3/E3), which can be useful for the diagnosis as early as between the 14th and 22nd weeks of gestation (Shackleton et al. 2007).

Mutations in the gene encoding P450 oxidoreductase are less frequent and result in a variant of a congenital adrenal hyperplasia involving combined deficiency of the cytochromes P450C17 and P450C21. The disease phenotype depends on the patient's gender. Most striking phenotypic features of affected girls are ambiguous genitalia at the time of birth, indicating intrauterine androgen excess. However, after birth virilization does not progress and amounts of circulating androgens are low or normal. The boys affected are sometimes born undermasculinized (Arlt et al. 2004). Maternal signs of virilization by the 23rd week of gestation, often associated with aromatase deficiency, have also been reported (Shackleton et al. 2004). MS allows the detection of PORD as early as in the 12th weeks of gestation (Reisch et al. 2013b). In the urine of pregnant mothers of fetuses with PORD, there is an increased excretion of the progesterone metabolite 5α-pregnane-3β,20α-diol (epiallopregnanediol), while physiologically pregnanediol (5β-pregnane-3α,20α-diol) constitutes the main metabolite of

progesterone. Remarkably, a rapid increase in the androgen metabolite androsterone can be observed in the first trimester, with a peak at around 20 weeks. Over the last 30 years, PORD has been repeatedly misdiagnosed, usually as variants of 17α-hydroxylase deficiency, due to insufficient number of plasma analytes measured by conventional techniques to unravel both 17α- and 21-hydroxylase deficiencies.

Postnatal urine steroid profile of a patient with PORD is one typically seen in 21-hydroxylase deficiency, with increased content of pregnanetriol, 17-OH-pregnanolone, and pregnanetriolone (17-OHP and 21-deoxycortisol metabolites), increased excretion of mineralocorticoid precursors (corticosterone metabolites), and a decreased excretion of active androgen metabolites (Shackleton and Malunowicz 2003).

3 Congenital Adrenal Hyperplasia – Newborn Screening

Defective steroid synthesis and metabolism may result from different forms of congenital adrenal hyperplasia, tumors, and altered peripheral metabolism of adrenocortical hormones, with adrenal insufficiency being a life-threatening disorder. Adrenal insufficiency in pediatric patients is mostly a result of primary adrenal failure. A prompt diagnosis and management are essential to implement successful treatment.

Congenital adrenal hyperplasia (CAH) is the most frequent (prevalence 1:150,000) inherited adrenal insufficiency and the second most frequent cause of primary adrenal insufficiency after autoimmune destruction. Clinical and biochemical manifestations of CAH forms are summarized in Table 2.

The most common form of CAH (95% of cases) is 21-hydroxylase deficiency (21-OHD) caused by a mutation in the *CYP 21A2* gene encoding the adrenal 21-hydroxylase. 21-hydroxylase is responsible for the conversion of 17-hydroxyprogesterone (17-OHP) to 11-deoxycortisol and of progesterone to deoxycorticosterone, which are the precursors for cortisol and aldosterone, respectively. The most apparent biochemical sign of 21-OHD is a significant elevation of 17-OHP concentration in the plasma and its metabolite in the urine. In over 40 countries, CAH screening programs are introduced into clinical routine to detect neonatal patients, especially males, before onset of symptoms. The screening process, however, is characterized by a high false-positive rate and a very low positive predictive value, especially in preterm infants when compared with term infants (Coulm et al. 2012). The immunoassays commonly used for the measurement of 17-OHP in the CAH screening produce a large number of false-positives due to the lack of specific antibodies for 17-OHP. Another significant problem is an elevation of 17-OHP during stress and illness. Moreover, there are many limitations for 17-OHP measurements in preterms due to a high activity of the fetal adrenal zone, delayed physiological maturation of 11β-hydroxylase, and poor kidney function. In contrast, steroid profiling with gas chromatography-mass spectrometry (GC/MS) in a urine spot or by liquid chromatography-mass spectrometry (LC/MS) in a dry blood spot allows the accurate and specific evaluation of adrenal steroid hormones by a simultaneous measurement of several hormones, which makes it a convenient diagnostic tool.

GC/MS is considered a highly specific and accurate method useful for a urinary steroid metabolite analysis. Hormonal imbalance caused by enzyme deficiencies results in a depletion of steroid products and the accumulation of upstream precursors, indicating the presence of a metabolic block. It has been clearly demonstrated that a relatively high urinary content of 17-OHP metabolites in preterm newborns is a consequence of diminished 11β-hydroxylase activity. Thus, it is not recommended to use urinary 17-OHP metabolites for the diagnostics of 21-OHD in preterms (Kamrath et al. 2014). Kamrath et al. (2016) have retrospectively assessed the diagnostic metabolite ratios for neonates and infants with and without 21-OHD using GC/MS, focusing especially on the glucocorticoid metabolism. The authors have measured urinary 17-OHP

Table 2 Clinical and biochemical manifestations of most frequent disorders affecting synthesis and metabolism of steroid hormones.

Disorder (deficient or defective enzyme; major tissue distribution of enzyme)	Clinical & biochemical findings	Symptoms			
Congenital adrenal hyperplasia (CAH)		Neonate	Infant	Adolescent	Adult
21-OHD (21-hydroxylase deficiency; adrenals, gonads)	Dehydration	±	±	±	±
	Various degrees of genital ambiguity in females	+	+	+	+
	Advanced somatic development (both genders)	N/A	−	−	+
	Short stature in adults	N/A	N/A	N/A	+
	Potassium (P)	↑	↑	N-↑	N-↑
	Sodium (P)	↓	↓	↓-N	↓-N
	Inappropriate natriuresis	+	+	±	±
	17-OH-progesterone (P)	↑↑	↑↑	↑↑	↑↑
	DHEA, androstenedione, and testosterone (P)	N-↑	↑	↑	↑
	Pregnantriol and androgen metabolites (17-keto-steroids) (U)	N-↑	↑	↑	↑
	Aldosterone and cortisol	↓	↓	↓	↓
	Plasma renin activity (PRA) (P)	↑	↑	↑	↑
	ACTH	↑	↑	↑	↑
	Various degrees of genital ambiguity in females (from clitoral enlargement to penile urethra)	+	+	+	+
	Advanced bone age	±	+	+	+
	Decreased fertility in males (low sperm count)	N/A	N/A	N/A	+
11β-OHD (11β-hydroxylase deficiency; adrenals)	Genital ambiguity in females	+	+	+	+
	Episodic vomiting	±	+	+	+
	Headache	±	+	+	+
	Hypertension	±	+	+	+
	Advanced somatic development (both sexes)	N/A	+	+	+
	Short stature	N/A	−	−	+
	Potassium (P)	↓	↓	↓	↓
	Sodium (P)	↑	↑	↑	↑
	Deoxycorticosterone, 11-deoxycortisol, androgens (P)	↑	↑	↑	↑
	Cortisol and aldosterone (P)	↓	↓	↓	↓
	Plasma renin activity (PRA) (P)	↓	↓	↓	↓
	ACTH (P)	↑	↑	↑	↑
	Various degrees of genital ambiguity in females	+	+	+	+
	Advanced bone age	±	+	+	+

(continued)

Table 2 (continued)

Disorder (deficient or defective enzyme; major tissue distribution of enzyme)	Clinical & biochemical findings	Symptoms			
Congenital adrenal hyperplasia (CAH)		Neonate	Infant	Adolescent	Adult
3β-HSD (3β-hydroxysteroid dehydrogenase type II deficiency; adrenals, gonads)	Dehydration	±	±	±	±
	Ambiguous genitalia (both sexes)	+	+	+	+
	Post-natal virilization (females)	N/A	N/A	+	+
	Potassium (P)	↑	↑	↑	↑
	Sodium (P)	↓	↓	↓	↓
	Δ^5 Steroids (17-OH-pregnenolone, DHEA) (P)	↑	↑	↑	↑
	Ratio Δ^5/Δ^4 steroids (P, U)	↑	↑	↑	↑
	Aldosterone, cortisol, sex hormones (P)	↓	↓	↓	↓
	ACTH (P)	↑	↑	↑	↑
	Various degrees of genital ambiguity in females	+	+	+	+
	Males: Hypospadia, undescended testes	+	+	+	+
	Females: Cliteromegaly	±	±	±	±
17-OHD (17-hydroxylase deficiency; adrenals, gonads)	Episodic vomiting	+	+	+	+
	Headache	N/A	N/A	N/A	N/A
	Hypertension	±	+	+	+
	Female external genitalia	+	+	+	+
	Lack of sexual development	N/A	N/A	+	+
	Alkalosis (B)	+	+	+	+
	Potassium (P)	↓	↓	↓	↓
	Sodium (P)	↑	↑	↑	↑
	Deoxycorticosterone, corticosterone (P)	↑	↑	↑	↑
	Cortisol, sex hormones, aldosterone (P)	↓	↓	↓	↓
	Infantile external genitalia in females	+	+	+	+
	Undescended testes in males	+	+	+	+
ILD (isolated 17,20-lyase deficiency; adrenals, gonads)	Male: various degrees of genital ambiguity	+	+	+	+
	17-OH-progesterone (P)	↑	↑	N -↑	N-↑
	Cortisol, aldosterone (P)	N	N	N	N
	DHEA, androstenedione testosterone (P)	↓	↓	↓	↓
	External genitalia in males: ambiguous	+	+	+	+
	Undescended testes	+	+	+	+
Glucocorticoid remediable aldosteronism (defective 11β-hydroxylase I/II; adrenals)	Headache	−	+	+	+
	Hypertension	+	+	+	+
	Potassium (P)	↓	↓	↓	↓
	Aldosterone (P)	↑	↑	↑	↑
	Aldosterone after dexamethasone (P)	↓	↓	↓	↓
	18-Oxocortisol (U)	↑	↑	↑	↑

(continued)

Table 2 (continued)

Disorder (deficient or defective enzyme; major tissue distribution of enzyme)	Clinical & biochemical findings	Symptoms			
Congenital adrenal hyperplasia (CAH)		Neonate	Infant	Adolescent	Adult
Apparent mineralocorticoid excess (11β-hydroxysteroid dehydrogenase type 2 deficiency; kidneys, adrenals, placenta)	Headache	–	+	+	+
	Hypertension responsive to low sodium diet	+	+	+	+
	Potassium (P)	↓	↓	↓	↓
	Tetrahydrocortisol (THF)/ tetrahydrocortisone (THE) ratio (U)	↓	↓	↓	↓
Cortisone reductase deficiency (defective 11β-hydroxysteroid dehydrogenase type 1; liver)	Hirsutism and other signs of androgen excess in females	–	–	+	+
	Cortisol (P)	N/A	N/A	N	N
	ACTH (P)	N/A	N/A	N	N
	Adrenal androgens (DHEA, androstendione) (P)	N/A	N/A	↑↑	↑↑
	All 17-ketosteroids (androsterone, etiocholanolone, DHEA) (U)	N/A	N/A	↑↑	↑↑
	Cortisol and cortisone metabolites (THF, THE) (U)	N/A	N/A	↑↑	↑↑
Cushing's syndrome	Weight gain, growth retardation, hirsuitism, obesity (BMI > 85th percentile),violaceous skin striae, acne, hypertension;	+	+	+	+
	Cortisol (P, U)	↑↑	↑↑	↑↑	↑↑
	Tetrahydrocortisone (THE)/ tetrahydrocortisol (THF) ratio (U)	↑	↑	↑	↑

N/A not applicable, *N* normal, *B* blood, *P* plasma, *U* urine, *ACTH* adrenocorticotropin hormone, *17-OHP* 17-hydroxyprogesterone, *DHEA* dehydroepiandrosterone

metabolites such as 17α-hydroxyprognanolone (17α-OHPN), pregnanetriol (PT), 15β,17-α-dihydroxypregnanolone (15β,17α-OHPN), and urinary pregnanetriolone (PTN), a metabolite of 21-deoxycortisol (21-DOF). They have also assessed the content of the most typical neonatal glucocorticoid metabolites, i.e., tetrahydrocortisone (THE) and 6α-hydroxyated form of cortisol metabolites such as 6α-hydroxy-THE (6αOH-THE), 6α-hydroxy-β-cortolone (6αOH-β-CTN), and 6α-hydroxy-α-cortolone (6αOH-α-CTN). The findings are that all the ratios, using PTN as the numerator and different combinations of the main urinary glucocorticoid metabolite as the denominator, clearly separate 21-OHD from non-21-OHD neonates and infants with 100% sensitivity and 100% specificity. The authors have also recommended PTN/6-α-hydroxytetrahydrocortisone (THE) ratio and PTN/(THE and 6α-hydroxyTHE) ratio as the two best indices for the diagnosis of 21-OHD. The conclusion is that a detailed analysis of glucocorticoid metabolism, especially the use of typical neonatal 6α-hydroxylates metabolites, can significantly improve the diagnostic utility of metabolite ratios.

Although GC/MS is still the 'gold standard' for steroid profiling, mainly in the urine, the diagnostic role of LC/MS or tandem MS constantly increases. LC/MS/MS is especially useful for neonatal screening, where the problem of small sample size is commonly encountered. Moreover, using a highly specific LC/MS/MS method for quantification of 17-OHP can reduce the number of unnecessary tests, the time needed for the diagnosis, and the anxiety of families and physicians. As the elevated level of 17-OHP is a common feature in all most common types of

CAH (21-OHD, 11-OHD, 3β-hydroxysteroid dehydrogenase deficiency, and 17α-hydroxylase deficiency), steroid profiling is necessary for a differential diagnosis between otherwise undistinguishable CAH subtypes. So far, few methods based on LC/MS/MS for the simultaneous quantification of several steroids in dry blood spots have been validated. Boelen et al. (2016) have developed an assay and provided gestational age-specific reference ranges for cortisol, cortisone, 11-deoxycortisol, 21-deoxycortisol, 17-hydroxyprogesteron, testostosterone, Δ^4-androstenedione, corticosterone, and 11-deoxycorticosterone in dry blood spots. They have also found that none of the steroids, except 21-deoxicortisol, were 100% specific in the CAH patients. Notably, 21-deoxicortisol is a pathological marker of 21-OHD CAH, produced from the accumulated 17-OHP *via* 11β-hydroxylation, the level of which falls into the reference range in patients suffering from 11β-OHD. Another assay for multiple steroids analysis has been presented by Kim et al. (2015) who simultaneously quantified seven steroids: cortisol, 17-hydroxyprogesteron, 11-deoxycortisol, 21-deoksycortisol, androstenedione, corticosterone, and 11-deoxycorticosterone. Notably, as deficiency in 21-hydroxylase results in increased levels of 17-OHP and androstenedione, simultaneous measurement of cortisol and androstenedione, along with 17-OHP, can provide an improved discrimination between truly affected and unaffected patients, especially in neonates under stress. A profiling panel including such steroid hormones as 11-deoxycortisol, 11-deoxycorticosterone, and corticosterone promotes a discrimination between CAH subtypes, especially among 21-OHD and 11β-OHD, as well as the differential diagnosis of 11β-hydroxylase 1 *vs.* 11β-hydroxylase 2 deficiency. In patients with 11β-hydroxylase 1 deficiency, 11-deoxycortisol and 11-deoxycorticosterone levels are typically very high (20–300 times over the upper limit of the reference range), while 11-deoxycortisol is only slightly or not at all elevated in patients with 11β-hydroxylase 2 deficiency.

4 Diagnosis of Premature Adrenarche

It is a challenging and daunting task to diagnose the disorders underlying premature adrenarche, precocious prepuberty, or hyperandrogenism in pediatric patients. As there are several rare conditions with similar symptoms, in most cases it is difficult to establish a definite diagnosis. Hyperandrogenism in children and adolescents is frequently associated with disorders in steroidogenesis, mainly with non-classic form of CAH and a disorder of peripheral metabolism of adrenal steroid hormones.

The non-classical form of CAH (NCAH) with 21-hydroxylase deficiency (NC21-CAH) is one of the most common autosomal recessive diseases. It affects *ca*. 0.3% of white population but with varied frequency depending on ethnic group (Bidet et al. 2009). In women with clinical symptoms suggesting hyperandrogenism, NCAH can be confirmed in 1–10% of patients. Patients with NCAH, in contrast to the classical form of CAH, have no symptoms at birth. Androgenic symptoms, single or complex, most often appear at puberty and include early pubarche in both sexes, acne, accelerated growth velocity and bone age advancement, and compromised final height.

The clinical features of NC21-CAH in children may be difficult to differentiate from those of the premature adrenarche and in postpubertal adults from the polycystic ovary syndrome (PCOS). Although the measurement of random 17-OHP concentrations usually enables the diagnosis of the classical form of CAH, the level of 17-OHP in individuals with NC21-CAH may be within the normal range. Thus, the acute ACTH stimulation test remains the gold standard to confirm decreased 21-hydroxylase activity. Accordingly, hormone content is measured in two blood samples: before and 30–60 min after synthetic ACTH is administered. The congruity of hormone content with genetic analyses suggest that mutations are likely to be identified on both alleles when the ACTH-stimulated 17-OHP

value exceeds 1500 ng/dL, but one should be aware that some, especially older, NC21-CAH patients, will demonstrate lower ACTH-stimulated 17-OHP levels between 1000 and 1500 ng/dL. An alternative diagnostic approach is based on urine steroid profiling. To diagnose NCAH, the excretion of 17-OHP (of adrenal-gonadal origin), 21-deoxycortisol (of adrenal origin, a specific marker of 21-hydroxylase deficiency) and metabolites of cortisol are estimated and diagnostic ratios are calculated. Importantly, the presence of cross-reacting steroids of fetal adrenal origin may hinder the interpretation of 17-OHP immunoassay results in preterm and term infants. The use of LC/MS/MS is thus highly beneficial in newborns, but the sensitivity and specificity of this technique makes it applicable also for 17-OHP determinations in children, adolescents, and adults.

Unexplained hyperandrogenism in girls without disorders of sexual development also can result from a rare milder form of non-classic CAH – steroid 11β-hydrocylase deficiency (11-OHD) (Nimkarn and New 2008). Early diagnosis and treatment implementation are essential to avoid severe long-term complications of hyperandrogenism and arterial hypertension. After excluding more common non-classic 21-OHD, baseline and stimulated 11-deoxycortisol tests should be performed to diagnose the specific type of non-classic 11-OHD. A non-classic 11-OHD is likely to be missed when relying on the standard immunoassay steroid hormone panel. As plasma 11-deoxycortisol immunoassay shows cross reactivity with 21-deoxycortisol, the urinary steroid profiling should be performed to diagnose rare forms of hyperandrogenism, such as 11-OHD and cortisone reductase deficiency. The implementation of LC/MS/MS for hyperandrogenism, including 11-deoxycortisol levels, overcomes the issue of cross reactivity and makes more invasive functional stimulation tests in children and adolescents redundant (Reisch et al. 2013a).

A number of steroid profiles have been developed over the past 10 years, which boosted the clinical usefulness of data when compared to the measurement of a single steroid. Plasma steroid profiling is especially helpful for differential diagnosis of non-classical forms of CAH, which may be indistinguishable using clinical presentation and sonography alone. Unfortunately, a complicated process of the production of calibrators and quality control samples that should be used in the performance of each multi-steroid assay limit the availability of steroid profiling. Moreover, although standardization of these methods to minimize analytical intra- and inter-laboratory variability and inaccuracy is highly desirable, it is also difficult to achieve. Currently, there are several commercial standardized kits in the market which enable simultaneous analyses of several hormones with high accuracy, e.g., SteroIDQ and MassChrom Steroid kits. They offer a standardized, quantitative steroid hormone detection by the assessment of 17 and 13 different steroid hormones, respectively. SteroIDQ assesses the level of aldosterone, androstenedione, androsterone, corticosterone, cortisol, cortisone, 11-deoxycorticosterone, 11-deoxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, dihydrotestosteron, estradiol, estrone, etiocholanolone, 17-α-hydroxyprogesterone, progesterone and testosterone. MassChrom Steroid kit, on the other hand, covers androsterone, 11-deoxycorticosterone, estrone and etiocholanolone measurements (AbsoluteIDQ Stero17 Kit 2017; Chromsystems MassChrom Steroids 2017). By covering a broad range of potential indications and simultaneous quantification of a broad range of steroid hormones, dysregulations in the steroid hormone biosynthesis can be detected early, enabling the physician a more comprehensive and faster differential diagnosis.

Besides non-classical forms of CAH, another reason for premature adrenarche is tumors of the adrenal cortex. These tumors occur rarely in both children and adults, representing only 0.2% of all cancers found in children and 0.02% of all cancers in the whole population. In children, tumors of the adrenal cortex are most often detected in the first decade of life, mostly before

the age of five. Although some tumors remain hormonally inactive, in a vast majority of cases affected children present clinical symptoms associated with excessive production of adrenal hormones. In these patients, the content of glucocorticoids, mineralocorticoids, and sex hormones can be elevated, in association with the three main clinical presentations, or in most cases a combination thereof: Cuching's syndrome, hypertension and hypokalemia, virilization and feminization. Among those three, virilization is the most common and occurs in over 50% of children. It is manifest as an increase in lean body mass, accelerated growth, acne, premature pubarche, hirsutism, and clitoris or penis enlargement. Another frequently observed disturbance is the presence of a mixed syndrome, including both virilization and hypercortisolism.

Notably, the intensity of steroidogenesis is not always the only feature which varies between healthy adrenal tissue and adrenocortical tumor, as in some cases tumor cells produce only steroid precursors of poor biological activity. However, clinical symptoms may still occur as a result of an increased tumor mass and overall high levels of steroid precursors, which affect target tissues either directly or after peripheral transformation to more biologically active derivatives, which is observed in case of androgen. Importantly, the presence of increased amounts of steroid precursors in the blood, along with clinical symptoms, may be mistakenly interpreted as a biochemical sign of CAH (Werder et al. 1994). It has been shown *in vitro* that tumor tissue can be characterized by low activity of many enzymes with a simultaneous, selective increase in the activity of one or several enzymes with high affinity to unusual substrates. The most common enzyme deficiency in adrenal cortex tumor cells are: 3β-hydroxysteroid dehydrogenase (3β-HSD) deficiency with increased excretion of DHA and DHEAS, and 11β-hydroxylase deficiency with increased production of 11-deoxycortisol and deoxycorticosterone (DOC). The analysis of steroid profiles in girls with virilizing tumors of the adrenal cortex by Małunowicz et al. (1995) have confirmed that there are different types of enzyme deficiencies in the adrenal cortex tumor tissue. In most cases, a massive secretion of metabolites of 3β-hydroxy-Δ^5-steroids, especially DHE and its derivatives such as 16 -α-hydroxydehydroepiandrosterone (16α-OHDHA), is observed. In addition, high levels of metabolites of pregnenolone (Δ^5PD) and 17α-hydroxypregnenolone (Δ^5PT) are found in urine samples. In that study, the profile of secreted metabolites confirmed 3β-HSD deficiency in tumor tissue. Moreover, many patients had an increased level of tetrahydro-11-deoxycortisol (THS), which provided evidence for the accompanying 11β-hydroxylase deficiency.

A detailed analysis of urinary steroid metabolites profile in children with virilizing tumors is of high diagnostic value. Large amounts of 3β-hydroxy-Δ^5-steroids dominating in steroid profile suggest virilizing adrenal tumors in prepubertals, but do not enable the definite biochemical diagnose of a tumor in children during puberty. Such a diagnosis should be confirmed with the dexamethasone inhibition test. On the other hand, when excessive secretion of 3β-hydroxy-Δ^5-steroid metabolites is accompanied with increased excretion of cortisol precursors, such as THS, tumor of the adrenal cortex should be suspected (Małunowicz et al. 1995).

Beside the aforementioned conditions, another disorder leading to hyperandrogenism is the altered metabolism of cortisol. Recent evidence suggests that obesity, insulin resistance, hypertension, polycystic ovary syndrome, and hyperthyroidism may all arise from defects in cortisol metabolism. The available methods enable the estimation of the whole panel of cortisol metabolites and also the investigation of hydroxysteroid dehydrogenase (HSD) activity, which altogether leads to a more thorough assessment of cortisol homeostasis.

Cortisol availability is controlled by complementary activities of two enzymes: 11-β-hydroxysteroid dehydrogenase type 1 (11β-HSD1, also known as cortisone reductase) and type 2 (11β-HSD2). 11β-HSD1 catalyzes the conversion of inactive 11-oxo glucocorticoid to

active 11β-hydroxyl forms, i.e., from cortisone (E) to cortisol (F) in humans and plays a pivotal role in the regulation of energy metabolism in the liver, adipose tissue, and skeletal muscles. The latter enzyme catalyzes the opposite reaction and is highly expressed in the placenta and kidney, protecting the mineralocorticoid receptors from glucocorticoid overload. A simultaneous use of chromatographic separation with spectrometric detection is an advanced technology used to separate these epimeric steroids, being particularly useful in the diagnostics of disorders such as cortisol reductase deficiency (CRD) (Krone et al. 2010). The lack of cortisol regeneration stimulates ACTH-mediated adrenal hyperandrogenism, which causes the onset of symptoms such as precocious pseudopuberty, hirsuitism, oligomenorrhea, and infertility (Lawson et al. 2011). A subtle local increase in cortisol concentration can be detected in a 24-h urine collection. To evaluate 11β-HSD2 activity cortisol/cortisone ratio is measured, while 11β-HSD1 activity is assessed based on the ratio of their metabolites. Biochemically, CRD can be diagnosed by the assessment of urinary cortisone and cortisol metabolites, by determining the ratio of tetrahydrocortisol (THF) + 5α-THF to tetrahydrocortisone (THE) and the ratio of cortols to cortolones. In CRD patiens, the THF+5αTHF/THE ratio is typically <0.1; the reference range is 0.7-1.2 (Tomlinson et al. 2004).

5 Monogenic Hypertensive Disorder Associated with Steroids Metabolism

Altered peripheral metabolism of cortisol can lead to hypertension as a leading clinical symptom, which occurs, e.g., in patients suffering from apparent mineralocorticoid excess (AME). AME is an autosomal recessive disease characterized by a low-renin hypertension associated with low aldosterone concentration, metabolic alkalosis, hypernatremia, and hypokalemia. The disorder is caused by a congenital defect in 11β-HSD2 activity, resulting in a decreased conversion of biologically active cortisol to inactive cortisone (Palermo et al. 2004). This defect makes cortisol to act as a ligand for the mineralocorticoid receptor, which leads to sodium retention, volume expansion, and hypertension (Ferrari 2010). The urinary steroid metabolite profile in patients with AME consists mostly of reduced metabolites of cortisol (5-β-tetrahydrocortisol (THF) and 5α-THF or allo-THF) with very low or undetectable tetrahydrocortisone (THE). The excretion of 5α-cortisol metabolites exceeds that of 5β-cortisol metabolites and results in a high urinary allo-THF/THF ratio suggesting an additional defect in 5β-reductase activity. The most prominent change in AME is increased THF + allo-THF/THE ratio, typically ranging from 3 to over 70, whereas in healthy subjects the ratio is approximately 1. In the past, the THF + allo-THF/THE ratio was used to diagnose AME. However, it has been suggested that this ratio provides only an index of global 11β-HSD activity within the body, i.e., primarily 11β-HSD1 activity in the liver and 11β–HSD2 in the kidney. It appears that renal 11β-HSD2 activity is better reflected by the measurement of plasma [11-^{3}H]-cortisol half-life and the ratio of urinary free cortisol/urinary free cortisone (UFF/UFE). Normal subjects excrete two to three-fold more UFE than UFF, reflecting a significant activity of renal 11β-HSD2. In AME, however, UFE excretion is virtually undetectable, resulting in a high UFF/UFE ratio. Although plasma cortisol half-life is prolonged (120–190 min in patients *vs.* 70–90 min in healthy subjects), the patients with AME are not cushingoid as the cortisol secretion rate falls to very low levels due to the intact negative feedback mechanism that maintains the cortisol's circulating concentration within the normal range despite its impaired metabolism.

Another disorder accompanied with hypertension as the prevalent clinical symptom, usually occurring early in life, is glucocorticoid remediable aldosteronism (GRA). The disease is characterized by acute exaggerated aldosterone

responsiveness to ACTH and a failure to show a normal decline in aldosterone content following chronic continuous ACTH infusion. The adrenal cortex in GRA produces large quantities of 18-oxygenated cortisol compounds, 18-oxocortisol (18-oxoF), and 18-hydroxycortisol (18-OHF). 18-oxoF and 18-OHF are so-called hybrid steroids due to enzymatic features of both zona glomerulosa and zona fasciculata steroids, exhibiting aldosterone and 17-hydroxylase activities, respectively. It remains unclear whether these compounds have sodium-retaining properties and contribute to the phenotypic variability of the disorder. Elevated levels of 18-oxoF and 18-OHF in a 24-h urine collection provide a highly sensitive and specific test to diagnose GRA. Overproduction of 18-oxoF and 18-OHF also can be observed in patients with aldosterone-producing adenoma (APA). However, these two conditions can be easily distinguished, as the levels of 18-oxoF and 18-OHF in GRA exceed the nom up to 20–30 times, compared to their modest elevation in APA. In addition, reduced 11β-hydroxylase activity and elevated levels of the mineralocorticoid 11-deoxycorticosterone have been reported in untreated individuals with GRA (Litchfield et al. 1995).

6 Diagnosis of Hypercorticism

Cortisol is a major circulating glucocorticoid in humans. Both excessive secretion and insufficient production can threaten patient's health and life, especially in young individuals. To confirm clinical suspicion of excessive or insufficient secretion of cortisol and to determine its etiology, a comprehensive panel of laboratory tests is required. However, the measurement of morning cortisol concentration in the serum, the evaluation of the circadian rhythm of cortisol, the low-dose dexamethasone suppression test, and the determination of free cortisol excretion in the urine are all burdened with inaccuracies of various sort (El-Farhan et al. 2017; Meikle 1982). Whereas immunoassays suffer from the interference of cross reactivity with cortisol A-ring metabolites and other steroids, LC/MS/MS overcomes these limitations, which gives accurate results with high selectivity and sensitivity. Although the cortisol content can be measured in many biological samples including the whole blood, plasma, serum, urine, saliva and hair, the routine clinical diagnostics of disorders of the hypothalamic-pituitary-adrenal axis has been dominated by the use of serum, saliva, and urine.

The serum cortisol quantification is an important test, especially in patients with Cushing's syndrome receiving metyrapone, which is 11-β-hydroxylase inhibitor used to block cortisol synthesis. Moreover, metyrapone is used by several countries for the diagnostics of adrenal insufficiency. The serum cortisol measurement by routine immunoassay is prone to interferences when the sample comes from patients receiving metyrapone. In these patients, inhibition of 11-β-hydroxylase causes a massive increase in cortisol precursors (e.g., 11-deoxycortisol) that can interfere in immunoassay and potentially mask the drug overdose-induced hypocortisolism. That has recently been recognized in the Endocrine Society's guidelines concerning the treatment of Cushing's syndrome (Auchus 2014). The guidelines recommended LC/MS/MS for the serum and urine cortisol quantification in the patients who receive metyrapone, as this methodology circumvents the issues encountered in immunoenzymatic tests. In addition, measurement of 11-deoxycortisol and cortisol supports the diagnostic interpretation of the dynamic function test in adrenal insufficiency in patients receiving metyrapone (McWhinney et al. 2010).

The measurement of urine free cortisol is recommended for the investigation of hypercortisolism, as it represents the non-protein bound cortisol freely filtered through the glomerulus. It is, however, recommended only after a 24-h urine collection, which may be troublesome in other than in-hospital settings. The utility of blood or saliva tests is limited, as they assess the cortisol level only at a particular time of the day. Moreover, some people find blood tests

stressful, which leads to increased cortisol release and a lower reliability of the testing.

Due to its lipophilic nature, serum free cortisol can passively diffuse into saliva *via* the acinar cells, independently of saliva flow rate. Therefore, akin to urinary free cortisol, salivary cortisol is a good surrogate of serum free cortisol content. The use of saliva for the cortisol measurement has an advantage over both urine and serum as saliva contains relatively fewer interfering compounds. In addition, saliva tests are non-invasive and saliva collection is simple and cost effective, which offers an advantage for both patients and physicians. Saliva testing is recommended as the first-line test for the diagnosis of Cushing's syndrome and it appears also useful the workup for adrenal insufficiency (Hawley and Keevil 2016). The non-invasiveness of saliva collection minimizes the stress-induced cortisol response, which facilitates the assessment of hydrocortisone replacement therapy and creation of day-curves, optimizing steroid replacement.

The measurement of total cortisol in the hair seems a future direction for LC/MS/MS method in the diagnosis of glucocorticoid-related disorders. As cortisol is deposited in hair shafts, it has been postulated that hair analysis may be useful to assess chronic exposure to cortisol. The test would support clinicians in the diagnosis of Cushing's syndrome. It could also be used to monitor hydrocortisone replacement therapy and to assess pathologies associated with chronic stress (Hawley and Keevil 2016).

Mass spectrometry appears helpful not only to confirm a persistently elevated level of serum or urinary cortisol, but to detect diagnostically important pathological metabolites of cortisol. The steroid profile of urine in patients with hypercortisolemia confirms the excessive secretion of all cortisol metabolites, including 6α-OHF and 20α-DHF, which are undetectable in healthy children's urine. A quantitative evaluation of secretion of all cortisol metabolites and its precursors in one analysis has a high discriminatory power. As a non-invasive method that does not require hospitalization and eliminates the stress-induced cortisol excretion, it seems a particularly superior screening test in the pediatric population.

7 Summary

Human steroid hormones and their metabolites have been traditionally quantified in biological samples by simple radioimmunoassay techniques. Nevertheless, immunoassays have considerable limitations, including the cross-reactivity between structurally similar molecules, suboptimal specificity, limited dynamic range, and a significant matrix effects (Allende et al. 2014). These limitations have been under underlined by the Endocrine Society in articles on the challenges of steroid determination (Rosner et al. 2013; Vesper and Botelho 2010; Rosner et al. 2007). The reliance on direct steroid immunoassays does not meet the up-to-date diagnostic and therapeutic standards concerning the identification and quantification of endogenous steroid molecules. These standards are congruous with the mass spectrometry-based assays that ensure the required accuracy of measurement to optimize the way patient care and therapy are provided (Handelsman and Wartofsky 2013; Wartofsky and Handelsman 2010).

Recently, a trend for exploring and mapping the entire metabolic pathways has become evident. The differentiation and quantification of unusual steroids during multi-analyte detection have become a necessity to accurately diagnose numerous pathological disorders. Chromatographic separation coupled to mass spectrometry fulfill this role and predominate in the analytic field of steroid metabolomics as these methods are capable of singling out a precise molecular pathology and thus enable the initiation of target-treatment in cases that would previously be undistinguishable when relying on immunoassay tests and clinical symptoms (Demkow 2010; Noppe et al. 2008).

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Discriminant Analysis of Intracranial Volumetric Variables in Patients with Normal Pressure Hydrocephalus and Brain Atrophy

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Abstract

A method was developed for the computerized volumetric assessment of the intracranial cerebrospinal fluid (CSF) distribution. The study involved 62 patients differentiated into two groups: with CSF resorption disorders (normal pressure hydrocephalus – 30 patients) and without CSF resorption disorders (various types of brain atrophy – 32 patients). The goal of the study was to ascertain whether the assessment, depending on the linear discriminant analysis of volumetric brain features, could be an effective tool differentiating the two groups. Volumetric measurements were performed using VisNow software. For each patient, five features were determined and subjected to discriminant analysis: CSF volume in the subarachnoid space and basal cisterns (SV), CSF volume in the intracranial ventricular system (VV), brain volume (BV), total intracranial CSF volume (FV), and total intracranial volume (TV). Discriminant analysis enables the achievement of a high percentage of correct classification of patients to the appropriate group determined on the result of a lumbar infusion test. The discriminator, based on three features: BV, SV, and VV, showed a complete separation of the groups; irrespective of age. The squared Mahalanobis distance was 70.8. The results confirmed the

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applicability of the volumetric method. Discriminant analysis seems a useful tool leading to the acquisition of a computer-aided method for the differential diagnosis of CSF resorption disorders.

Keywords
Computer-aided diagnosis • CSF resorption disorders • Linear discriminant analysis • Normal pressure hydrocephalus • Normotensive hydrocephalus • Volumetric assessment

1 Introduction

One of the most important adverse symptoms of aging is cognitive impairment. It is a process that the modern medicine cannot prevent or treat. The exception is a pathological process known as normal pressure hydrocephalus (NPH). Since cerebrospinal fluid (CFS) resorption disturbances are the fundamental cause of NPH, the disease can be treated with the implantation of different kinds of external CSF draining devices. In the decision-making concerning the initiation of treatment, the major difficulty lies in the differential diagnosis of NPH and various types of dementia collectively described as brain atrophy (BA) (Kiefer and Unterberg 2012; Relkin et al. 2005).

There is as yet no clinically accepted algorithm for the differential diagnosis between NPH and BA. Nor is there a consent on the most appropriate criteria for qualifying a patient for surgery valve implantation (Williams and Relkin 2013; Mori et al. 2012; Krauss and Halve 2004). Usual neurological, neuropsychological, and imaging examinations are not enough to unambiguously set the differential diagnosis of a disease with multiple uncharacteristic symptoms. According to many authors, the gold standard for determining the presence of a resorption disorder, is the lumbar infusion test (Brean and Eide 2008; Bech-Azeddine et al. 2005; Czosnyka et al. 2005).

The infusion test has an edge over other ways of assessing the outcome of implantation treatment for CFS shunting as it gives an 80% conformity of a positive outcome. For comparison, CSF tap test has about 50% conformity with the outcome (Aoki et al. 2015; Kahlon et al. 2002). However, both tests are highly invasive, burdened by complications. In recent years, research centers have been working on alternative little or non-invasive indirect methods to assess the CSF shunting (Gosche et al. 2001; Sutton et al. 1986), which give specificity and sensitivity close to that of the infusion test. To this end we also have developed the following methods:

- analysis of changes in parameters of somatosensory evoked potentials (Jurkiewicz et al. 1991);
- analysis of gait and balance disorders (Czerwosz et al. 2013; Czerwosz et al. 2008);
- mathematical elaboration assessing the planimetric distribution of CSF in individual intracranial compartments (Marszałek et al. 1997; Jurkiewicz 1996). This method enables the determination of a number of fluid points (pixels) of 3–8 jHU density (Hounsfield 1980) in selected sections of CT images; and
- volumetric measurements based on VisNow software (Szczepek et al. 2015; VisNow 2011) of CSF volume in the intracranial ventricular system and in the subarachnoid spaces.

In the methods we chose to employ the multivariate linear discriminant analysis (LDA) (Devijver and Kittler 1980; Anderson 1958) that in our opinion yields the best possible statistical confirmation of the volumetric results obtained. The goal of the present study was to answer the question of whether a computerized volumetric method of CSF distribution assessment in the

intracranial brain compartments, employing LDA, would effectively differentiate between normal pressure hydrocephalus and brain atrophy.

2 Methods

The study was performed in accordance with the guidelines of the Helsinki Declaration for Human Research and the protocol was accepted by the Bioethics Committee of Warsaw Medical University (permit no. KB/64/2014). Informed consent was obtained from all the patients involved in the study.

The patients were referred to the hospital for differential diagnosis of a brain disorder. A common clinical feature was an enlargement of cerebral ventricles. For further investigation, we accepted only the patients with ventriculomegaly; having the Evans index >0.3 (Ng et al. 2009). Since non-invasive neurological and neuropsychological examinations failed to establish an unambiguous diagnosis, a lumbar infusion test was conducted, with the cut-off resorption resistance (R) >11 mmHg/ml/min as indicative of a resorption disorder. On the basis of the test, patients were divided into two groups: NPH, a CSF resorption disorder, R > 11 mmHg/ml/min and BA, R < 11 mmHg/ml/min. There were 62 patients in all. Thirty two patients were diagnosed with BA, mean age of 70 ± 5 years, and 30 patients with NPH, mean age of 58 ± 6 years. Patients with suspected 'arrested hydrocephalus' and those with 'mixed hydrocephalus', who alongside a resorption disorder had signs of brain atrophy, were not included into either group. For the correctness of discriminant analysis we purposefully gathered groups of about the same size, which equalizes the *a priori* probabilities. The patients were not matched in terms of age and gender, and the establishment of a relative incidence of both NPH and BA was not the purpose of the study.

Neuropsychological examinations were based on a set of tests developed in the Neurosurgery Clinic of the Second Faculty of Medicine of Warsaw Medical University in Poland (Marszałek et al. 1997). Volumetric measurements in CT scans of the head were made using VisNow software developed in the Laboratory of Visual Analysis of the Interdisciplinary Center for Mathematical and Computational Modelling at Warsaw University in Poland (VisNow 2011). We used a semi-automatic procedure for the intracranial volume segmentation and volumetric analysis consisting of two basic steps: 1/ volume normalization obtained from DICOM data and 2/ multi-step regional growing/distance mapping of brain tissue, ventricles, and extraventricular fluid volume.

Firstly, acquired data were linearly interpolated to a regular 0.5 mm grid and were de-noised with a two-step anisotropic de-noiser preserving the contour surfaces. Then, segmentation process began with marking the intracranial volume characterized by voxel density over 64 HU (Hounsfield 1980), using a flood fill algorithm, starting from the center of the head volume. Brain tissue was segmented as the area of intracranial volume with the voxel density above 30 HU. Finally, the ventricles were separated from the CSF area with an interactive marking of flood fill starting point(s). The visual segmentation process used renders the images that more truly reflect both brain layers and volumes in perturbed intracranial geometry, particularly concerning the brain ventricles, as opposed to the atlas-type segmentation prone to a substantial distortion. The following volumetric variables, thereafter called features, were subjected to analysis:

- CSF volume in subarachnoid spaces and basal cisterns (SV);
- CSF volume in the intracranial ventricular system (VV);
- brain volume (BV);
- total intracranial CSF volume (FV = SV + VV);
- total intracranial volume (TV = SV + VV + BV).

Discriminant analysis was used to define the classification rule and to classify patients of known diagnosis (Devijver and Kittler 1980;

Anderson 1958). The method enabled the differentiation of two classes, i.e., groups of NPH and BA patients, on the basis of a set of features, i.e., SV, VV, BV, FV, and TV variables, and the calculation of a discriminant function from these features.

The Lilliefors test showed that all the variables had a Gaussian distribution, except VV in the NPH group for which a small skewness appeared with the median shifted rightward of the mean. Having a substantial number of patients, and since the data distributions were compact and almost separable between the groups, we chose to use parametrical *t*-tests and a linear discriminant analysis (LDA), in which the discriminant function is a linear combination of a set of features.

Discriminant analysis enabled the classification of patients and its comparison with the results of an infusion test taken as a reference. The diagnosis had to accurately assign the patients to a particular class, either NPH or BA group, with the 'ground truth' reliability; the term that refers to the accuracy of the training sets for supervised learning techniques in the pattern recognition systems and classification algorithms. Classification effectiveness was specified as the percentage of correct and incorrect classified patients. In case of two groups, the number of patients belonging to the first or second group and are classified with the use of the classification rule to the first or second group can be counted. These four figures of patients create a so-called two-way contingency table. The mutual dependence of the table figures was evaluated with the Chi squared (χ^2) test (Yates 1934). The classification rule should maximizes the ratio of between-group variation to within-group variations. The measure of the obtained classification rule is the Mahalanobis distance (Mahalanobis 1936).

The main goal of discriminant analysis was the automated diagnosis of new patients of unknown diagnosis and the prediction of their being part of either NPH or BA group. An accurate diagnosis is possible only when a stable discriminant rule is created and when that rule proves effective in the classification of patients with known diagnosis. In determining the classification rule, a rotation of the coordinate system takes place, which made it possible to maximize the distance of the group centroids and thereby produced a more accurate separation of NPH and BA patients. At the same time, the dimension of the measuring space was reduced. When two classes are considered (NPH and BA), the method yields a single one-dimensional discriminant function.

Statistical analysis was performed with a commercial Statistica v9.0 package (StatSoft; Tulsa, OK). The *k*-nearest neighbours (k-NN) (Duda et al. 1973) analysis was performed using proprietary software (Jóźwik 1994).

3 Results

The mean values of the intracranial volumetric variables were significantly different in the BA and NPH patients as assessed with a *t*-test. Likewise, discriminant analysis on a single feature turned out a very sensitive statistical tool for differentiation between the two groups of patients. In this analysis a single volumetric variable was considered a discriminant function. The percentage of correct classifications was counted as a ratio of correctly qualified patients to the total number of patients in classification matrix concerning either group. The squared Mahalanobis distance between centroids of a group is also presented. The distance was calculated for single volumetric variables. It enables the differentiation between almost identical discriminant powers of particular discriminant rules (Table 1).

Figures 1a–d illustrate the distribution of separate volumetric variables drawn in a two-dimensional coordinate system, with the single straight lines drawn at an angle of about 45° representing a graphic visualization of linear discriminant function. There is a clear differentiation of the intracranial volumes of fluid compartments. The CSF volume in the ventricular system (VV) was evidently greater than that

Table 1 Statistical analysis of volumetric variables in patients with brain atrophy (BA) and with normal pressure hydrocephalus (NPH)

Measure	BA n = 32	NPH n = 30	*t*-test p	Classification matrix			Squared Mahalanobis distance	k-NN misclassification rate (E_r)
					NPH	BA		
SV (cm^3)	141 ± 21	53 ± 7	<0.0001	NPH	30	0	31.7	0.00
				BA	0	32		
VV (cm^3)	111 ± 13	173 ± 15	<0.0001	NPH	29	1	19.4	0.20
				BA	0	32		
BV (cm^3)	1,131 ± 29	1,268 ± 42	<0.0001	NPH	30	0	14.5	0.00
				BA	2	30		
FV (cm^3)	253 ± 23	225 ± 17	<0.0007	NPH	23	7	1.9	0.22
				BA	10	22		
TV (cm^3)	1,385 ± 31	1,493 ± 37	<0.0001	NPH	27	3	10.0	0.40
				BA	0	32		

SV CSF volume in subarachnoid spaces and basal cisterns
VV CSF volume in intracranial ventricular system
BV brain volume
FV total intracranial CSF volume
TV total intracranial volume

in the subarachnoid spaces and basal cisterns (SV) in NPG patients compared with BA patients (Fig. 1b).

It is worth noting that these functions are one-dimensional, each point on the two-dimensional chart represents a single patient and is replaced by a single value calculated from the discriminant function equation (Table 2). This is a graphic representation of a reduction of space dimension, with a simultaneous rotation of the coordinate system.

Figure 2 illustrates a three-dimensional coordinate system: brain volume (BV) *vs*. CSF volume in the intracranial ventricular system (VV) *vs*. CSF volume in the subarachnoid spaces and basal cisterns (SV). Patients from the NPH and BA groups are presented, and the position of average values (centroids of the group). From the centroids, a perpendicular line is drawn in the direction of each of the planes, its intersection with the plane determines a projection of the centroid on the plane. These projections are shown in Figs. 1b–d.

The results of linear discriminant analysis for five sets of volumetric variables measured are presented in Table 2. Four discriminant functions (LDA 1, LDA 2, LDA 3, and LDA 4) are determined for a two-dimensional coordinate system and one (LDA 5) for a three-dimensional BV, SV, VV system. This precisely corresponds to the distribution of data (i.e., the scattergrams of volumetric variables) illustrated in Figs. 1 and 2. Discriminant function equation defines the value of this function for single patients. For each of the five functions (LDA 1–5), a two-way matrix was determined, followed by Chi^2 statistics for each 2 × 2 matrix and a *p*-value (Table 2). In both two-dimensional (LDA 1–4) and three-dimensional (LDA 5) coordinate systems of volumetric variables the assignment of patients to either NPH or BA group turned out fully correct.

The discriminant analysis of volumetric variables of features provided almost identical results, amounting to a 100%. Therefore, it was necessary to use an additional measure, a Mahalanobis distance, in the discriminant evaluation. The squared Mahalanobis distance between the average values (centroids) of the groups, determined for each functions, is presented in the last column of Table 2. The greatest distance value of 70.8 was achieved for LDA 5, a three-dimensional coordinate system, with the discriminant equation containing three volumetric variables (features): SV, BV, and VV.

Histograms of LDA 5 values were calculated for 32 BA patients and 30 NPH patients. A

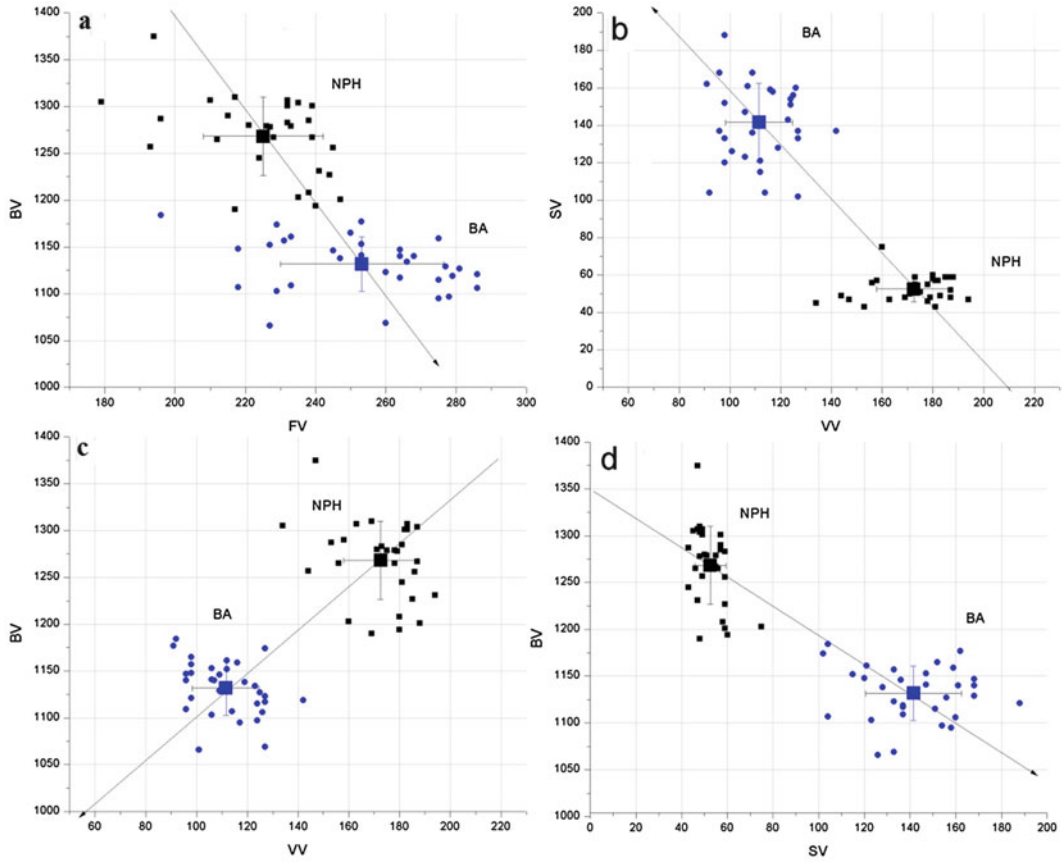

Fig. 1 Distribution of separate volumetric variables in a two-dimensional coordinate system in patients with brain atrophy (BA) and with normal pressure hydrocephalus (NPH). *Straight lines* drawn at an angle of about 45° represent a graphic visualization of linear discriminant function for each variable; (**a**) BV *vs.* FV, (**b**) SV *vs.* VV, (**c**) BV *vs.* VV, (**d**) BV *vs.* SV
BV brain volume, *FV* total intracranial CSF volume, *SV* CSF volume in subarachnoid spaces and basal cisterns, *VV* CSF volume in intracranial ventricular system

negative value of the LDA 5 variable for the NPH group and a positive value for the BA group are presented in Fig. 3. An outstanding separation of the patients of the two groups is noticeable.

4 Discussion

The diagnosis of NPH, which justifies a decision to undertake a surgical approach, is based on a set of invasive and non-invasive examinations. The decisive criterion is resorption resistance (R) ascertained by the infusion test, a gold standard approach providing the greatest clinical reliability. For the purpose of discriminant analysis conducted in the present study, we considered this reliability to be the 'ground truth'. The results of a lumbar infusion test may be disease- or age-specific, e.g., in subarachnoid hemorrhage or long-term illness (Czosnyka et al. 2011; Albeck et al. 1998). In the literature, R values in NPH patients usually vary between 11 and 18 mmHg/ml/min (Kahlon et al. 2005; Boon et al. 1997; Borgesen et al. 1992).

In our experience, a set of results consisting of R >11 mmHg/ml/min, it varied between 12 and 15 mmHg/ml/min in the majority of patients, full-blown Hakim's triad, ventricular enlargement, and a positive neuropsychological

Table 2 Linear discriminant analysis (LDA) of volumetric variables in patients with brain atrophy (BA) and with normal pressure hydrocephalus (NPH)

LDA	Features – original measured variables	Discriminant function equation	Centroid BA	Centroid NPH	Classification matrix			Chi^2	p	Squared Mahalanobis distance between centroids
						NPH	BA			
LDA 1	BV, FV	LDA1 = −0.028*BV − 0.00002*FV + 33.54	1.85 ± 0.81	1.97 ± 1.17	NPH	28	2	50.76	0.00001	14.5
					BA	0	32			
LDA 2	SV, VV	LDA2 = −0.049*SV + 0.043*VV − 1.167	3.37 ± 1.24	3.60 ± 0.66	NPH	30	0	58.06	0.00001	48.7
					BA	0	32			
LDA 3	BV, VV	LDA3 = −0.065*VV − 0.023*BV + 37.3	3.47 ± 0.87	3.70 ± 1.12	NPH	30	0	58.06	0.00001	51.4
					BA	0	32			
LDA 4	BV, SV	LDA4 = −0.051*BV + 0.013*SV − 10.59	3.07 ± 1.17	3.27 ± 0.77	NPH	30	0	58.06	0.00001	40.3
					BA	0	32			
LDA 5	BV, SV, VV	LDA5 = 0.034*SV − 0.050*VV − 0.017*BV + 24.105	4.07 ± 1.06	4.34 ± 0.92	NPH	30	0	58.06	0.00001	70.8
					BA	0	32			

BV brain volume
FV total intracranial CSF volume
SV CSF volume in subarachnoid spaces and basal cisterns
VV CSF volume in intracranial ventricular system

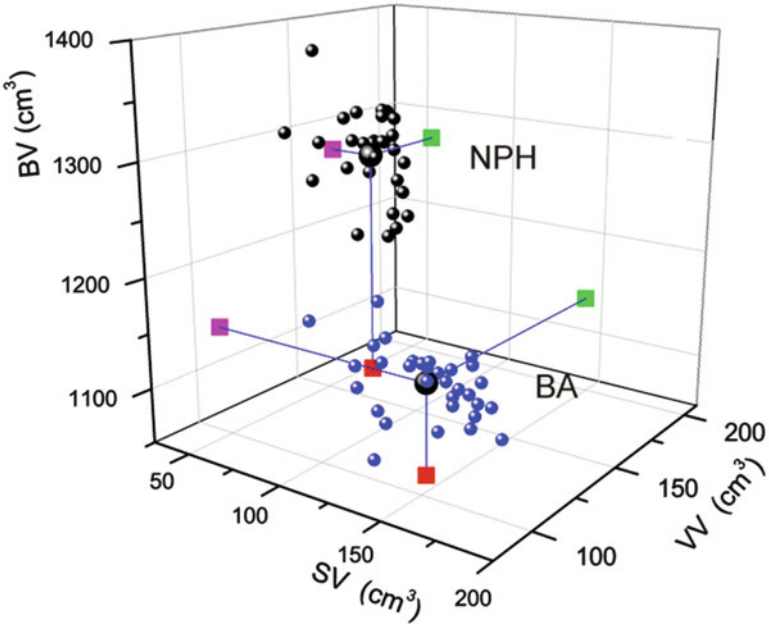

Fig. 2 Distribution of single variables of volumetric measurements in patients with brain atrophy (BA) and with normal pressure hydrocephalus (NPH) in a three-dimensional coordinate system of discriminant functions: BV *vs*. VV *vs*. SV
BV brain volume, *VV* CSF volume in intracranial ventricular system, *SV* CSF volume in subarachnoid spaces and basal cisterns

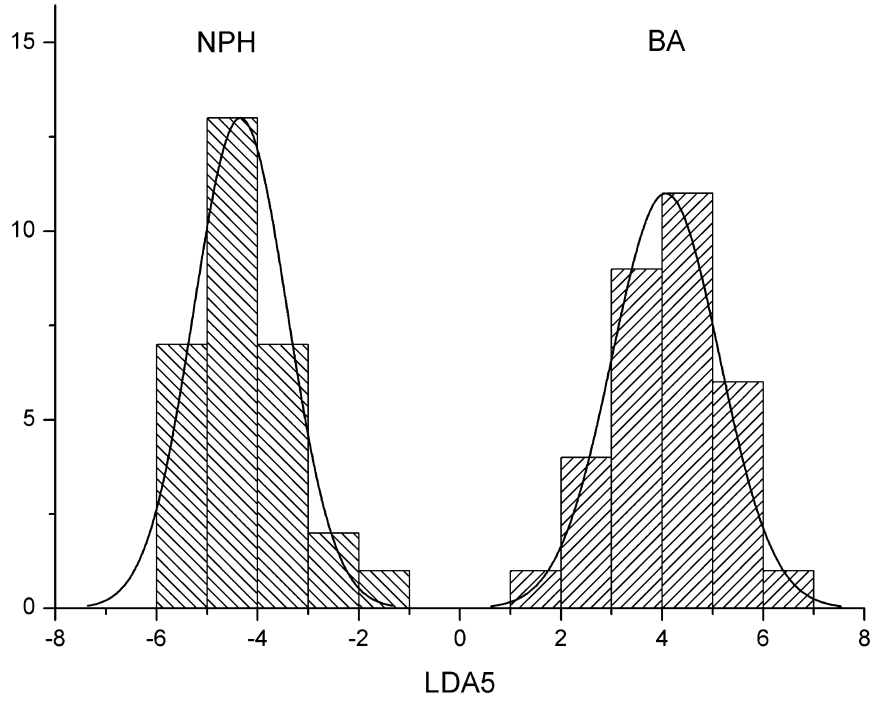

Fig. 3 Histogram of values of linear discriminant function LDA 5 for the volumetric variables BV, SV, VV in patients with brain atrophy (BA) and with normal pressure hydrocephalus (NPH)
BV brain volume, *SV* CSF volume in subarachnoid spaces and basal cisterns, *VV* CSF volume in intracranial ventricular system

examination warrants the implantation of a shunt valve. The present findings demonstrate that the volumetric workup clearly differentiates between CSF volumes in NPH and BA, as compared by a *t*-test. In addition, we confirmed the diagnostic power in NPH and BA patients of the volumetric evaluation proposed with another statistical classifier, a *k*-nearest neighbours method (k-NN). The smallest misclassification rate (E_r), with the greatest percentage of correct classifications, appeared when comparing the two variables: CSF volume in the subarachnoid spaces and basal cisterns (SV) and the brain volume (BV). A greater misclassification rate appeared when the following features were taken into consideration: total intracranial volume (TV), total intracranial CSF volume (FV), and CSF volume in the intracranial ventricular system (VV) (Table 1).

It is worth noting that CSF volume in the subarachnoid spaces and basal cisterns (SV) is the most useful variable for differentiating patients with and without CSF resorption disorders in both classification methods used. Jurkiewicz (1996) has drawn attention to this fact in a study on the differential diagnosis of hydrocephalus and brain atrophy based on the distribution of fluid pixels in specific intracranial compartments. The number of fluid pixels increased in the ventricular system or in the subarachnoid spaces and basal cisterns in NPH and BA, respectively. That observation was unambiguously confirmed in the present study.

Ishii et al. (2006) have developed brain volumetric software (AVSIS) that makes it possible to automatically measure regional CSF. In further studies these authors have discussed the issue of employing volumetry for determining CSF volume in the intracranial spaces in three groups of subjects: hydrocephalus, Alzheimer's disease, and healthy individuals (Ishii et al. 2013). The results of that and some earlier comparative evaluations (Ishii et al. 2008; Kitagaki et al. 1998) are in line with the findings of the present study.

The differentiation of NPH and Alzheimer's disease with the segmentation method of white matter, grey matter and the CSF distribution in specific central fluid compartments has also been studied by Serulle et al. (2014). The authors have evaluated MRI scans of NPH patients, who responded positively to shunt implantation, Alzheimer's patients, and of healthy subjects. A graphical separation of the patient groups visible in a two-dimensional chart of the grey matter volume *vs.* the intracranial ventricular volume is worthy of note. The distinguishing of AD from NPH was based on the binary logistic regression model and it reached close to 94%.

Discriminant analysis of volumetric measurements has certain limitations. To create a classification rule, so-called 'learning sample' was used consisting of patients with known diagnosis set on a 'ground truth' level of reliability. These patients also became 'testing sample' used for testing the classification method. Further, reliability of a discriminant evaluation could be weakened due to a small number of patients in the 'learning sample', which is identical to the 'testing sample', especially when the number of patients is about the same as that of features, i.e., variables measured. This kind of risk does not apply to the volumetric evaluation in the present study, since the number of patients was many times greater than the maximum number of variables, 62 *vs.* 5, respectively. There is an apparent need to extend the evaluation by making a cross-validation of results in which the 'testing sample' will be different from the 'learning sample'. Currently, a very high classification correctness, even for simple variables, may be due to the fact that no patients with diagnosed comorbidities were included into the study groups. It seems reasonable to enrol patients with borderline or uncertain diagnosis into the 'testing sample'. Statistical distribution of any feature deviating from normal distribution can also influence the reliability of a discriminant evaluation. In the present study, data distributions were close to normal, symmetrical, and of comparable variances. The charts of patients' values, concerning both NPH and BA, did not overlap to any significant extent, which enabled the use of a linear discriminant evaluation.

Aoki et al. (2015) have confirmed that discriminant evaluation enables the effective

non-invasive prognosis of outcomes in NPH patients treated with the implantation of a brain shunt. Discriminant analysis has since long been used in the assessment of EEG signals as a method of reducing the amount of data in the power spectrum analysis (Blinowska et al. 1981). A significant reduction of data takes place concerning both the number of bytes generated in a unit of time and the number of channels (electrodes). The evaluation enables an effective reduction in the dimension of data space and it enhances the separation of classes, unachievable with classical statistical methods. However, results of Aoki et al. (2015) are affected by EEG measurements from multiple leads and a large number of features (variables) obtained while calculating the power spectrum data. Such issues make a good repeatability of data obtained in a small number of patients doubtful while examining bigger cohorts of patients.

The present study indicates that subjecting volumetric features to discriminant analysis is a sensitive diagnostic procedure in brain disorders. CSF volume in subarachnoid spaces and basal cisterns (SV), in intracranial ventricular system (VV), and the brain volume (BV) perfectly classify patients to either NPH or BA group. The weakest differentiating volumetric feature was the total intracranial CSF volume (FV), which gave the same diagnostic yield a *t*-test or *k*-NN classification. This is understandable considering that FV is a sum of SV + VV, and these two volumes assume the opposite values in the NPH and BA groups.

Patient age appreciably differed in the present study. The NPH patients were significantly younger, mean age 58 $\pm$ 6 years, from the BA patients, mean age 70 $\pm$ 5 years. To check the possible effect of patient age on the results we made a selection of patients, rejecting the younger patients from the NPH group and older patients form the BA group. This procedure caused a loss of a significant age difference between the two groups; NPH – 63.6 $\pm$ 2.6 years (13 patients) and BA – 66.1 $\pm$ 3.8 (12 patients). The elaboration carried out now on a limited number of 25 patients fully confirmed the discriminative power of volumetric features subjected to discriminant analysis in the diagnosis of NPH and BA on par with the full group of 62 patients. Further, the discriminator correctly differentiated the patients rejected in the age-equalization procedure, which caused that these patients found themselves in the 'testing sample' but not in the 'learning sample'. In addition, we rechecked the potential interference of age using Pearson's correlation method, separately for NPH and BA group; finding no feature in correlation with age.

In conclusion, we submit that discriminant analysis, establishing the classification rule for patients with known diagnosis, and then classifying patients with unknown diagnosis, eventually may resolve the problem of non-invasive differential diagnosis in patients with and without brain resorption disorders. We believe we have shown that application of discriminant analysis greatly enhances the capability of computer-aided diagnosis of brain pathologies.

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Conflicts of Interest The authors declare no competing interests in relation to this article.

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Hoffa's Fat Pad Abnormality in the Development of Knee Osteoarthritis

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Abstract

Over the past two decades, many hypotheses have been put forward to explain the cause of knee osteoarthritis. Scientific reports bring up the role of adipose tissue in the activation of the inflammatory mechanisms, which is a characteristic feature of osteoarthritis natural history. Adipose tissue produces and releases cytokines, interleukins, and growth factors by means of paracrine, endocrine, and autocrine mechanisms. Hoffa's fat pad (infrapatellar adipose tissue) plays a viable role in the initiation and progression of osteoarthritis due to its role in the activation and release of pro-inflammatory mediators. The degenerative joint disease is considered an inflammatory process. Therefore, in this article we overview the importance of Hoffa's fat pad in the development and progression of osteoarthritis.

Keywords

Adipose tissue • Adipokine • Cytokines • Inflammation • Leptin • Osteoarthritis

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1 Introduction

Over the past two decades, many hypotheses have been put forward to explain the cause of knee osteoarthritis. According to mechanical theory, the repetitive micro-trauma occurring throughout the life span leads to the degeneration of articular cartilage. Another theory links osteoarthritis with metabolic disorders such as hormone and inflammatory markers imbalance. Scientific reports bring up the role of adipose tissue in the activation of the inflammatory mechanisms, which is a characteristic feature of the osteoarthritis natural history (Ehling et al. 2006). The adipose tissue is an active endocrine organ associated with appetite regulation, tissue insulin sensitivity, and bone metabolism (Fantuzzi 2005), but also secrets pro-inflammatory mediators and growth factors (Chaldakov et al. 2003; Coppack 2001). Hoffa's fat pad is a stripe of adipose tissue interposed between the joint capsule and the synovium in the anterior knee compartment. It fills the space between patellar tendon, femur, tibia, and menisci. The pad protects the joint against mechanical damage, acting as a shock absorber. It is richly innervated by a posterior articular branch of the tibial nerve and receives blood supply from the synovial membrane (Kohn et al. 1995). The information on the role of Hoffa's fat pad in the development of osteoarthritis is rather scarce. However, there are many reports associating the anterior knee pain syndrome with changes occurring in the infrapatellar pad, such as hypertrophy, inflammation, and fibrosis (Bohnsack et al. 2009; Magi et al. 1991). Macule et al. (2005) have reviewed seventy patients who underwent a total knee arthroplasty due to advanced osteoarthritis. The fat pad was resected during the procedure. During a six-months follow-up time, the patients reported no pain in the operated joint. These and other authors suggest that Hoffa's fat pad might be a source of chronic knee pain relayed through a rich network of C nerve endings, containing substance P, innervating this tissue (Witowski and Wagrowska-Danilewicz 1999).

2 Inflammatory Mechanisms in Osteoarthritis

Natural history and progression of a degenerative joint disease is multifactorial considering both etiology and inducing mechanisms. The disease stems from a combination of improper joint biomechanics and genetic, pro-inflammatory, and humoral factors (Guilak et al. 2004; Spector and MacGregor 2004; Ghosh and Smith 2002). Instability due to ligamentous injury is a well-known cause of joint dysfunction. A loss of muscle strength, peripheral neuropathy, and obesity have a significant impact on the knee biomechanics (Englund et al. 2004). Surgical removal of a torn meniscus may also alter the biomechanics, leading to articular cartilage degeneration. A cartilage damage and mechanical joint overload associated with abnormal joint motions leads to the activation of chondrocyte and osteoblast mechanoreceptors. In response to mechanical stimuli, the cells activate the inflammatory processes, which results in release of mediators causing further articular cartilage destruction (Berenbaum and Sellam 2008; Chowdhury et al. 2008; Gosset et al. 2006; McGlashan et al. 2008).

Genetics also influences the inflammatory processes and their dynamics. Approximately 39-65% of patients suffering from knee osteoarthritis may have the predisposing genetic factors that affect the cartilage matrix composition and chondrocyte signaling molecules (Spector and MacGregor 2004). The inflammatory process is part of osteoarthritis, influencing both onset and dynamics of disease progression. The cardinal findings of inflammation such as swelling, increased temperature, pain on motion, or rigidness are clearly seen. The pro-inflammatory mediators interleukins (IL) and tumor necrosis factor (TNF) alpha are released from chondrocytes, bone, synovial membrane, and other tissues surrounding the joint. Those substances activate metalloproteinases (MMPs), resulting in articular cartilage degradation due to the activation of catabolic gene expression (Goldring and Berenbaum 2004; Goldring 2000b). The mediators outlined above activate

prostaglandin E2 due to expression and activation of cyclooxygenase-2 (COX-2), microsomal prostaglandin E synthase-1 (mPGES1), and phospholipase A2 (sPLA2). These mediators trigger the synthesis of nitric oxide as they directly stimulate inducible nitric oxide synthase (iNOS) and activate other pro-inflammatory cytokines such as IL-1, IL-6, IL-8, leukemia inhibitory factor (LIF), IL-17, and IL-18. The inflammatory processes and their products inhibit differentiation of chondrocytes and type-II collagen synthesis (Goldring and Goldring 2007; Goldring 2000a). Transforming growth factor beta 1 (TGF-β1) secreted by macrophages located in the joint synovial membrane induces the osteophyte formation (Blom et al. 2004; van Lent et al. 2004). The inflammatory processes affecting the subchondral bone trigger excessive chondrocyte differentiation and induce paracrine signaling (Sanchez et al. 2005a, b; Guévremont et al. 2003).

3 Association Between Overweight and Development of Osteoarthritis

Overweight and a high body mass index are significant risk factors for the development of degenerative joint disease (Berenbaum and Sellam 2008). Altered kinematics of joints that bear substantial loads may be the cause of onset and progression of osteoarthritis (Andriacchi and Mundermann 2006; Andriacchi et al. 2004). However, joints that are not subject to excessive load bearing, such as joints of upper limbs, also are at higher risk of osteoarthritis in the overweight population compared to patients within the normal weight range (Yusuf et al. 2010; Kalichman et al. 2009; Oliveria et al. 1999; Groble et al. 2008; Cicuttini et al. 1996). In terms of symptom reduction, body weight loss is less significant than body fat loss (Toda et al. 1998). Aside from the amount of body fat, its location also influences the development of joint degeneration. Abdominal obesity carries a higher risk of knee and hip arthroplasty. It is associated with higher secretion of pro-inflammatory mediators from visceral fat. Subcutaneous fat, on the other hand, is characterized by a much lower activity (Fain 2006). The adipose tissue has an established role as the endocrine organ affecting the joint tissue metabolism.

4 Hoffa's Fat Pad Cells as Modifiers of Knee Osteoarthritis Course

White adipose tissue has long since been regarded as the explicit tissue for energy storage. However, in 1994, along with the discovery of leptin, white adipose tissue has gained new functions, including behavioral modifications, immune system modifications, and a modulation of inflammatory processes (Fain 2006; Hang et al. 1994). The tissue consists of highly specialized and well-differentiated cells and contains adipocytes, fibroblasts, leucocytes, macrophages, and other cells that play a role in inflammation. A collagen and elastic fiber scaffold holds these cells together, and the epithelial cells, nerves, and vessels penetrate them. Hoffa's infrapatellar fat pad, along with the synovial membrane, constitutes a very delicate and sensitive structure that is richly innervated with C-fibers releasing substance P (Bohnsack et al. 2005 Dye et al. 1998). In patients with osteoarthritis, both the number of C-fibers and the number of medium sized and large fibers containing substance P greatly increases (Lehner et al. 1985). Such a dense innervation is suggestive of a role the infrapatellar pad plays in the pain stimulus sensing in osteoarthritic patients. Secretion of substance P leads to vasodilation and migration of immune cells. The process results in swelling, tissue ischemia, and partial necrosis. All of these mechanisms also cause changes in Hoffa's pad structure and metabolism. According to Lowson and Steere (1985), infrapatellar fat pad edema observed in Lyme disease might be a result of inflammatory and degenerative joint changes. Ischemia induces a release of neurotropic growth factors that, in turn, activate substance P secretion into the intercellular space. This auto-stimulation mechanism

is likely responsible for chronic inflammation of Hoffa's fat pad and progression of degenerative joint disease (Lehner et al. 1985). In the experimental models, substance P activates NO release in the synovial membrane in rheumatoid arthritis (O'Shaughnessy et al. 2006). NO affects IL-1 beta, TNF alpha, and nuclear factor kappa B signaling; the cytokines that may oxidize the adjacent cells. Substance P also has a direct effect on fibroblast activation and thus on the composition of extracellular matrix (Lowson and Steere 1985).

The adrenergic nervous system produces anti-inflammatory agents (e.g., noradrenaline) and endogenous opioids that inhibit pain perception and reduce secretion and activity of substance P. In the healthy synovial membrane, the ratio of sensory fibers containing substance P fibers to adrenergic nerves is close to 1:1. In patients suffering from rheumatoid arthritis, this ratio may be as high as 8:1 (Weidler et al. 2005). Similar values have been reported for Hoffa's fat pad in patients suffering from anterior knee pain after total knee arthroplasty (Lowson and Steere 1985). An increased number of sensory fibers containing substance P influences the dynamics and development of degenerative joint disease.

5 Adipose Tissue as Endocrine Organ

Adipose tissue produces and releases cytokines, interleukins, and growth factors by means of paracrine, endocrine, and autocrine mechanisms. All of these substances are found in the synovial fluid and have a direct effect on the articular cartilage and synovial membrane metabolism (Dumond et al. 2003; Schäffler et al. 2003). The immune system cells contained in the adipose tissue are responsible for the stimulation and production of pro-inflammatory mediators, except for leptin and adiponectin that are released mostly from adipocytes (Hang et al. 1994). Leptin is a key substance in the development of osteoarthritis, as its direct joint injection stimulates the formation of proteoglycans and growth factors. However, recent studies have refuted this theory, demonstrating that leptin stimulates the secretion of IL-1 beta and thus enhances the MMP activity and inflammatory processes (Vuolteenaho et al. 2009; Iliopoulos et al. 2007; Otero et al. 2003; Simopoulou et al. 2007). Leptin may also act synergistically with interferon-gamma in the activation of NO synthase, using the IL-1 pathway (Presle et al. 2006), and may facilitate the activation of macrophages, neutrophils, dendritic cells, natural killers, and Th1 cells (Matarese et al. 2007).

Adiponectin is an adipokine that has a well-documented role in decreasing the risk of obesity and cardiovascular diseases, but it may also act as a pro-inflammatory mediator in joint diseases (Gomez et al. 2009). Adiponectin induces MMP-1 activity and triggers IL-6 formation in the synovial membrane fibroblasts; the cells having a receptor for adiponectin (Tang et al. 2007). Chondrocytes exposed to adiponectin produce IL-6, MMP-3, MMP-9, and a monocyte chemoattractant protein-1 (MCP-1) (Lago et al. 2008). The adipose tissue-specific secretory factor (ADSF) and nicotinamide phosphoribosyl transferase (NAMPT) are also members of the adipokine family. ADSF increases after joint injuries and the substance itself triggers the formation of pro-inflammatory cytokines and has an influence on the loss of proteoglycans in the articular cartilage. ADSF joint injection in mice causes a condition resembling osteoarthritis (Gomez et al. 2009; Lee et al. 2009). NAMPT also exerts a pro-inflammatory effect on the synovial membrane fibroblasts and chondrocytes (Gosset et al. 2008; Brentano et al. 2007). The mechanism of action of adipokines is not exactly clear. Leptin and ADSF may stimulate bone formation, while adiponectin may have an opposing action on this process (Reid 2008). Adipose tissue synthesize and releases these and a spate of other pro-inflammatory mediators that take part

in the development of the degenerative joint disease (Hang et al. 1994; Toussirot et al. 2007).

6 Hoffa's Fat Pad Releases Pro-inflammatory Mediators in the Knee Joint

Adiponectin and ADSF concentrations are lower, while leptin concentration is greater, in the synovial fluid compared with those in the serum (Simopoulou et al. 2007; Chen et al. 2006). Leptin concentration in the synovial fluid associates with the severity of knee osteoarthritis (Ku et al. 2009). However, increased permeability of blood vessels in the inflamed synovial membrane cannot explain the appearance of adipokines, since ADSF content is lower than that of leptin in the joint despite a similar molecular weight (Shine et al. 1991; Wallis et al. 1987). Synovial fluid contains more leptin in women than in men suffering from knee osteoarthritis. The discrepancy might be due to gender-related hormonal effects; the synovial fluid/serum leptin ratio is different in men and women (Simopoulou et al. 2007). Women also tend to have a greater content of ADSF, but not of adiponectin, in the synovial fluid.

In patients suffering from knee osteoarthritis, Hoffa's fat pad and synovial fluid contain significant quantities of FGF-2, VEGF, TNF alpha, and IL-6. However, there is no association between the cytokine content in the synovial fluid and infrapatellar fat pad. In both synovial membrane and Hoffa's pad, there is a similar distribution of FGF-2 and VEGF (Ushiyama et al. 2003). Cell lines collected from Hoffa's pad produce and secrete large quantities of adiponectin and leptin, but the greatest leptin secretion takes place in the cell lines obtained from osteophytes in osteoarthritis (Simopoulou et al. 2007).

The formation of pro-inflammatory factors appears in different parts of the joint such as articular cartilage, synovial membrane, menisci, osteophytes, and also in Hoffa's fat pad. In obese patients, formation and release of IL-6, IL-6 receptors, and adiponectin are higher in Hoffa's fat pad compared with the subcutaneous adipose tissue. A feature of the infrapatellar fat pad is a lower expression of genes responsible for fat metabolism. Studies indicate that Hoffa's fat pad has a character of an independent organ with its own specifics (Distel et al. 2009).

7 Summary and Conclusions

Hoffa's fat pad plays a viable role in the initiation and progression of osteoarthritis by means of activation and release of pro-inflammatory mediators. As a degenerative joint disease is of inflammatory character, it seems reasonable to determine the importance of Hoffa's pad in the development and progression of osteoarthritis. The association between obesity and joint degeneration consists not only of distorted biomechanics, but also of the adipose tissue ability to activate the inflammatory process. The infrapatellar pad, due to its location in the knee, has a direct influence on the composition of synovial fluid and thus on the other elements forming the joint. The experimental animal models and human trials cast more light on the mechanisms of immune cells migration into the inflammation site. Pro-inflammatory cytokines activating the pain-transmitting substance P fibers induce the anterior knee pain. A conjoined action of immune and nervous systems, along with the adipose tissue, exerts its direct effect on Hoffa's fat pad and on the formation of chemokines, cytokines, and growth factors. All of these effects influence the function and metabolism of the articular cartilage and synovial membrane. Considering the data above reviewed, it seems important to conduct further investigations on the entwined relations between the nervous system, the immune system, and the adipose tissue.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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