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Neuroscience and Respiration

Mieczyslaw Pokorski *Editor*

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Preface

The book series *Neuroscience and Respiration* presents contributions by expert researchers and clinicians in the field of pulmonary disorders. The chapters provide timely overviews of contentious issues or recent advances in the diagnosis, classification, and treatment of the entire range of pulmonary disorders, both acute and chronic. The texts are thought as a merger of basic and clinical research dealing with respiratory medicine, neural and chemical regulation of respiration, and the interactive relationship between respiration and other neurobiological systems such as cardiovascular function or the mind-to-body connection. The authors focus on the leading-edge therapeutic concepts, methodologies, and innovative treatments. Pharmacotherapy is always in the focus of respiratory research. The action and pharmacology of existing drugs and the development and evaluation of new agents are the heady area of research. Practical, data-driven options to manage patients will be considered. New research is presented regarding older drugs, performed from a modern perspective or from a different pharmacotherapeutic angle. The introduction of new drugs and treatment approaches in both adults and children also is discussed.

Lung ventilation is ultimately driven by the brain. However, neuropsychological aspects of respiratory 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 respiratory health is undeniable. Scientists accept a powerful psychological connection that can directly affect our quality of life and health span. Psychological approaches, by decreasing stress, can play a major role in the development and therapy of respiratory diseases.

Neuromolecular aspects relating to gene polymorphism and epigenesis, involving both heritable changes in the nucleotide sequence, and functionally relevant changes to the genome that do not involve a change in the nucleotide sequence, leading to respiratory disorders will also be tackled. Clinical advances stemming from molecular and biochemical research are but possible if the research findings are translated into diagnostic tools, therapeutic procedures, and education, effectively reaching physicians and patients. All that cannot be achieved without a multidisciplinary, collaborative, bench-to-bedside approach involving both researchers and clinicians.

The societal and economic burden of respiratory ailments has been on the rise worldwide leading to disabilities and shortening of life span. 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 books published in this series will assume a leading role in the field of respiratory medicine and research and will become a source of reference and inspiration for future research ideas.

I would like to express my deep gratitude to Mr. Martijn Roelandse and Ms. Tanja Koppejan from Springer's Life Sciences Department for their genuine interest in making this scientific endeavor come through and in the expert management of the production of this novel book series.

Opole, Poland Mieczyslaw Pokorski

Volume 17: Advances in Clinical Science

The book presents the latest developments in clinical medicine, particularly involved with pulmonary care and diseases. Emphasis is placed on the role of childhood obesity, often a result of the ill effects of an unhealthy diet and nutritional deficits, in shaping propensity for inflammatory pathologies, allergies, immune deficiency and respiratory and cardiovascular sequelae in adult life. Oxidative damage, caused by not full well understood cellular biochemistry, when unchecked by antioxidative rescue mechanisms, takes toll on a respiratory health. The book underscores the need to consider the complexity of mutual interactions of pathophysiological processes, which calls for tailoring the management strategies depending on the subgroups which patients belong to, be it obesity, children or elderly. The chapters also tackle biological diseases with genetic underpinnings. The application of genetics to identify the molecular alterations or mutations will serve well both diagnostics and targeted optimization of treatment; the poignant exemplar being the histological subtypes of lung cancer. The book provides a source of current facts and trends in clinical research and practice.

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Prevalence of Olfactory Impairment in Granulomatosis with Polyangiitis

K. Zycinska, M. Straburzynski, A. Nitsch-Osuch, R. Krupa, M. Hadzik-Błaszczyk, M. Cieplak, and K. Wardyn

Abstract

Granulomatosis with Polyangiitis (GPA) is a rare disease of unknown origin. It may damage all organs and systems, even olfactory and taste sense. The aim of the study was to determine the sense of smell in patients with GPA and to identify factors related to disease course, activity, and duration, which may be associated with olfactory dysfunction. The comparison of olfactory function screening scores with Sniffin' Sticks standardized norms showed that 74 % of the investigated patients had olfactory dysfunction. The olfactory performance was diminished in all parts of Sniffin' Sticks test: threshold scores 4.4 *vs.* 7.1 ($p = 0.007$); odor discrimination 9.0 *vs.* 11.9 ($p = 0.008$); and olfactory identification 9.8 *vs.* 12.2 ($p = 0.011$) in the GPA patients *vs.* control subjects, respectively. Scores acquired during all three parts of the test were combined to assess the TDI-score. The median TDI-score in the GPA group (27.5) was significantly lower than that in the control group (32.0) ($p = 0.002$). Active nasal and paranasal sinus inflammation in GPA leads to olfactory dysfunction, the patients are often unaware of. The dysfunction is permanent and does not abates along with decreasing intensity of the inflammatory process. GPA therapy should include recommendations on nutrition, personal hygiene, and food poisoning prevention.

Keywords

Anosmia • Granulomatosis with polyangitis • Odor discrimination test • Olfactory identification test • Parosmia • Sinusitis • Sniffin' Sticks test

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1 Introduction

At least 1 % of general population is anosmic, and about 5–8 % have a reduced olfactory function. Many people with reduced olfactory sensitivity are not aware of it. Using appropriate diagnostic tools or measurements of event-related potentials,

quantitative smell disorders such as anosmia or hyposmia, can be differentiated from normal olfactory function. However, some patients not only suffer from quantitative olfactory disorders, but also experience qualitative olfactory dysfunction, termed dysosmia or olfactory distortion. The latter disorders can be divided into parosmia (also called troposmia) and phantosmia. Typically, a vast majority of patients describe these parosmic experiences as unpleasant sensations (Göktas et al. 2010; Laudien et al. 2009; Ohashi et al. 1992). Granulomatosis with polyangiitis (GPA) is a rare disease of unknown origin. It may damage all organs and systems, but most often it affects the upper or lower respiratory tract and kidneys. The involvement of the olfactory system in the course of GPA has been reported by several authors, although there is still little evidence concerning the character of chemosensory disruption. The possible mechanisms of altered olfactory function may include: paranasal sinus and nasal involvement (rhino-sinusitis, bone structure damage, and granulomatous or atrophic changes in the mucous membrane), central or peripheral neural involvement (focal ischemic changes and cranial nerve damage), and medications. All chemical senses, i.e., olfaction, taste, and intranasal trigeminal perception, are mediated by the cranial nerves, and two of them, olfaction and intranasal trigeminal function, are altered by chronic rhino-sinusitis. As GPA may affect both upper airways and cranial nerves, the chemosensory function may be affected as well in these patients. Therefore, the aim of the present study was to determine the sense of smell in patients with GPA and to identify factors related to disease course, activity, and duration, which may be associated with olfactory dysfunction.

2 Methods

The study was approved by a local Ethics Committee of Medical University of Warsaw, Poland. Forty three patients with GPA (F/M – 31/12, mean age 47.7 ± 12.8 SD, range 18–73 years) from the Vasculitis Outpatients Clinic of the Czerniakowski Hospital in Warsaw, Poland, were included in the study. The diagnosis of GPA was established using the widely accepted criteria of the American College of Rheumatology and the European League Against Rheumatism (Felson et al. 2011). The control group consisted 21 - age-matched healthy volunteers. The procedures for all GPA patients included: medical history taken with the Multi-Clinic Smell and Taste (MCST) questionnaire (Nordin et al. 2003), physical examination with anterior rhinoscopy, supported by nasal endoscopy when rhinoscopy failed to show signs of GPA, determination of selected biochemical and serological parameters necessary for confirming the diagnosis, computed tomography of paranasal sinuses, questionnaire assessment of disease activity and extent using the Birmingham Vasculitis Activity Score (BVAS), the Disease Extent Index (DEI), the Vasculitis Damage Index (VDI) (Merkel et al. 2009; Seo et al. 2009; Mahr et al. 2008; Stone et al. 2001), and the assessment of olfactory function using the Sniffin' Sticks Extended Test for either side and bilaterally (Hummel et al. 2007). The procedure for the control group included: medical history with MCSTQ, physical examination with anterior rhinoscopy, and smell examination using the Sniffin' Sticks Extended Test.

The Sniffn' Sticks is a validated chemosensory function test based on the pen-like odor dispensing devices. The test consists of 3 units designed for olfactory threshold evaluation, identification and discrimination. Samples were presented in the felt-tip pens filled with 4 ml of liquid odorants or odorants dissolved in propylene glycol. For odor presentation, the cap was removed by a technician for approximately 3 s and the sample was placed 2 cm in front of the nostrils. The pens were presented at intervals to prevent olfactory desensitization. The olfactory threshold was defined with increasing concentration of n-butanol, strongest (1) of 4 % to weakest (16) of 1.22 ppm. The exact score is determined by presenting different concentrations of odor in predefined order. The lower the concentration identified by the patient, the higher is the threshold score (max. 16 points). In the olfactory discrimination test, the subject is asked to pick up a sample of different smell out of three samples; there were 16 combinations tested with points attributed to each correct answer (Table 1). A third part of the test determines the subject's capability of identifying everyday odors

Table 1 Sniffin' Sticks olfactory discrimination test

No	Odorant (1 pen)	Compared odorants (2 pens)
1	Octyl acetate (orange)	Cinnamaldehyde (cinnamon)
2	n-butanol (sweets)	2-Phenylethanol (flowers)
3	Isoamyl acetate (banana)	Anethole (anise)
4	Anethole (anise)	Eugenol (cloves)
5	Geraniol (flowers)	Octyl acetate (orange)
6	2-Phenylethanol (rose)	Isoamyl acetate (banana)
7	D-limonene (limes)	Fenchone (camphore)
8	L-carvone (spearmint)	D-carvone (caraway)
9	L-limonene (pine)	Citronellal (lemon)
10	2-Phenylethanol (flowers)	D-menthol (peppermint)
11	D-carvone (caraway)	Geraniol (flowers)
12	n-butanol (sweets)	L-limonene (pine)
13	Citronellal (lemon)	Linalool (flowers)
14	Pyridine (fish)	L-limonene (pine)
15	Eugenol (cloves)	Cinnamaldehyde (cinnamon)
16	Eucalyptol (camphor)	Leather

Table 2 Sniffin' Sticks olfactory identification test

No	Odor	No	Odor
1	Oranges	9	Garlic
2	Leather	10	Coffee
3	Cinnamon	11	Apples
4	Peppermint	12	Cloves
5	Bananas	13	Pineapple
6	Lemon	14	Rose
7	Liquorice	15	Anise
8	Terpentine	16	Fish

(Table 2). The task is to pick up a correct name for the sample presented out of 4 suggested answers (16 trials in this test). The final result was presented as a sum of the three parts of the test: threshold, discrimination, and identification (TDI score). The results may fall in one of the tree categories depending on the number of points in the TDI score:

- ≥31 – normosmia (normal olfactory performance),
- 15–29 – hyposmia (deficient olfactory performance),
- ≤14 – anosmia (no functional olfactory performance).

Table 3 Olfactory dysfunction in GPA patients

Olfactory function screening	n = 43	%
Normosmia (TDI >30)	11	26
Hyposmia (TDI 16–30)	22	51
Anosmia (TDI <16)	10	23

GPA granulomatosis with polyangiitis, *TDI* threshold, discrimination, and identification score

The results are presented as means ± SD. Normality of data distribution was checked with the Shapiro-Wilk test. Differences between the GPA and control groups were compared with the Mann-Whitney U and F tests.

3 Results

Sinonasal involvement was seen in 93 % of patients. The mean DEI, VDI, and BVAS values were 8.3 ± 2.9, 13.6 ± 6.4, and 11.9 ± 7.8, respectively. The mean time elapsed from the diagnosis of GPA (Td) was 11.9 ± 7.9 months, time from the diagnosis to the study (Ts) 13.5 ± 11.9 months. In 79 % of patients we observed suppurative and bloody nasal discharge, which was also the first disease symptom in 37 % of patients. Only had 8 patients in the GPA group undergone paranasal sinus and nasal surgery. The surgical procedures included ethmoidectomy, nasal septum plastic surgery, removal of nasal polyps, and the management of a suspected orbital tumor originating in the paranasal sinuses. Physical examination showed the evidence of crusting in 47 % of patients, nasal septum perforation (30 %), atrophy of nasal mucosa (16 %), and a saddle nose deformity (16 %). Computed tomography of paranasal sinuses of the investigated patients was reviewed for disease signs and their location. Inflammatory changes usually occurred in the maxillary sinus (72 %) and their degree was measured using the Lund-Mackay system for chronic rhinosinusitis, with the mean score of 5.8 ± 6.1 points. Apart from paranasal sinus opacification, tomography showed nasal septum perforation and widespread bone damage, reaching in some cases the orbit wall.

A comparison of olfactory function screening scores with the Sniffin' Sticks standardized norms showed that 74 % of GPA patients were dysfunctional (Table 3). Olfactory performance

Table 4 Threshold, discrimination, and identification (TDI) score

	GPA	Control	p
TDI left nostril	21.0 ± 9.2 (3–36)	28.3 ± 5.2 (20–36)	0.002
TDI right nostril	22.0 ± 9.5 (4–35)	29.5 ± 4.7 (20–40)	0.004
TDI bilateral	23.2 ± 10.3 (6–38)	31.2 ± 5.3 (22–40)	0.001

Values are means ± SD (minimal-maximal)

was diminished in all parts of the Sniffin' Sticks Test. Threshold scores were significantly lower in the GPA patients compared with the healthy subjects 4.4 ± 3.4 *vs.* 7.1 ± 3.0, ($p = 0.007$), respectively. Likewise, odor discrimination and olfactory identification tests provided lower scores in the patients than in the healthy subjects; 9.0 ± 3.9 *vs.* 11.9 ± 1.6, $p = 0.008$, and 9.8 ± 3.8 *vs.* 12.2 ± 2.0, $p = 0.011$, respectively. The combined TDI score, for either nostril and bilaterally was significantly lower (Table 4).

Data analysis defined a relation between olfactory function scores and factors related to the patients' medical history. The results show the associations between the olfactory function threshold and patients' age ($p = 0.034$) and also the elapse of time from the diagnosis to the study ($p = 0.008$). Patients complaining of parosmia were characterized by a significantly worse olfactory performance ($p = 0.028$). We also found a correlation between the physical examination and Sniffin' Sticks Test results. Patients with crusting, pathological granulation, nasal polyps, nasal septum perforations, and a bone damage on rhinoscopy or endoscopy had a greater higher olfactory dysfunction ($p < 0.05$). The next part of the analysis concerned the relationship between olfactory function results and medical imaging. There was a significant, although moderate, correlation between rhinsosinusitis in computer tomography and olfactory function screening results; Lund-MacKay score comported with lower Sniffin' Sticks Test results ($p < 0.05$). Bone damage and nasal septum perforation shown in computer tomography images were also related with decreased chemosensory parameters ($p < 0.05$).

4 Discussion

The present study demonstrates that 73 % in GPA patients suffered from olfactory dysfunction. There is evidence of only two other studies focusing on chemosensory functions of GPA patients. Laudien et al. (2009) have conducted the first olfactory function screening in GPA patients. Seventy six patients underwent the Sniffin' Sticks Screening Test. A comprehensive study (Sniffin' Sticks Extended Test) was carried out only in 4 patients with most advanced olfactory dysfunction. Olfactory dysfunction was found in 18 % of cases. Only 95 % of participants displayed the upper respiratory tract involvement. The disease activity in the study outlined above was relatively low and of short duration compared with that in the present study. The test used by Laudien et al. (2009) is only approximate in character and should be treated as a preliminary step for comprehensive study, which is of a higher diagnostic value. It is worth noting that there is a correlation between the screening test and results of a comprehensive study. Göktas et al. (2010) have applied a much more comprehensive approach to testing chemosensory functions. Apart from using a reliable method of smell and taste screening, the authors also assessed function of the trigeminal nerve and the retronasal olfaction. A shortcoming of that study, however, was a small size of the group investigated, which makes the assessment of results difficult, over-representation of post-nasal surgery patients (67 %), and the patients' age (median 57 years). Nonetheless, more than half of the patients in that study were found to display signs of olfactory dysfunction. The study also showed dysfunction of taste and trigeminal

nerve; however, the authors provide no information about the norms assigned to the results (no control group). The results obtained in the present study confirm that olfactory dysfunction is more common in patients with increased crusting, pathological granulation and a higher Lund-MacKay score. In other words, active inflammation (rhinosinusitis) in GPA is related to olfactory dysfunction. On the other hand, unlike the Laudien et al.'s screening (2009), no correlation was seen between olfactory function and sinusitis symptoms displayed by patients, such as nasal obstruction, discharge, lower olfactory function, or facial pain and pressure. It is possible that olfactory dysfunction in the absence of symptoms of rhinosinusitis is related to systemic medications received by patients or subclinical inflammation of the upper respiratory tract. This notion finds support in Hoffman et al.'s (1992) observation that some patients do not have respiratory symptoms, although bronchoalveolar lavage shows neutrophilic inflammation in the lung. Nevertheless, it seems that rhinosinusitis plays a key role in GPA olfactory dysfunctions. Of the nasal diseases, rhinosinusitis and polyps are the most important cause of olfactory disorders, which seems to be a result of immunologic processes rather than blockage of air flow. In rhinosinusitis, the epithelium shows signs of lymphocyte and macrophage activity.

Cytokine secretion may lead to the damage of olfactory neurons and their apoptosis (Ge et al. 2002). Given the lack of data from olfactory epithelium biopsy in GPA patients, one cannot draw a conclusion that the immunologic mechanism plays a key role in olfactory dysfunctions in this group, even though immunologic components are crucial in the GPA disease itself. Bone damage seen during the physical examination and nasal septum perforation visible in computed tomography images are related to olfactory disorders. Both are signs of a GPA-related inflammation underwent in the past. Their association with olfactory dysfunction may be a proof that GPA changes lead to permanent olfactory dysfunction, which does not withdraw when the inflammatory process is constraint. GPA may also be related to central and peripheral nervous system involvement, therefore it seems possible that GPA olfactory disorders are also connected to this involvement. Although the olfactory nerve, the first cranial nerve, is seen more as an extension of the central nervous system than as a peripheral nerve in the strict sense, opinions have been voiced that damage to the nerve may be caused by granulomatous vasculitis followed by mononeuritis multiplex. In the presently investigated group, only did 12 patients display symptoms of central nervous system involvement, which was not statistically related to olfactory dysfunction. Laudien et al. (2009) support the notion that isolated involvement of the olfactory nerve in the course of GPA seems unlikely. In the present study, test results did not yield a statistically significant relation between olfactory dysfunction perceived by patients, reported in the MCTS questionnaire, and Sniffin' Sticks screening. This would suggest that contrary to Laudien et al. (2009) and Göktas et al.'s (2010) opinions, the patients are often unaware of having olfactory disorders. In this context, it is worth noting that some other authors also confirm the opinion about the lack of correlation between subjectively perceived smell dysfunction and olfactory function test results (Landis et al. 2009). However, in the present study many patients (33 %) reported parosmia in the form of episodic sensing of unpleasant smells. This symptom is strongly associated with olfactory disorders.

In the present study, time from the diagnosis GPA to inclusion in the study was related to isolated instances of a higher olfactory threshold. Laudien et al. (2009) have had similar observations, although they tested the ability to identify smells. The diagnosis is related to the commencement of cyclophosphamide and glucocorticosteroid treatment. It would seem that prolonged therapy could have an impact on GPA patients' olfactory function. This is a significant observation, since medication seems to affect the sense of smell in an antagonistic way: chemotherapeutic agents may worsen the patients' olfactory function (Wickham et al. 1999), while systemic corticosteroids improve the sense of smell in rhinosinusitis

(Doty and Bromley 2004). All patients of the present study received the same combined form of therapy consisting of systemic cyclophosphamide and glucocorticosteroid, which does not allow for the assessment of differential effects of either medication on olfactory function. In Laudien et al.'s (2009) study, patients underwent different forms of therapy and the authors did not show any association between therapy and olfactory dysfunction. Those authors also failed to show any association between inflammatory (CRP, OB, WBC, and d-dimer) or serological (ANCA – anti-neutrophil cytoplasmic autoantibody) indicators and olfactory dysfunction. The lack of such an association was confirmed in our present study (data not shown). This study primarily aimed at showing olfactory dysfunction as a symptom accompanying GPA. An analysis of available sources shows that smell impairment may affect patients. Persons with anosmia and hyposmia (Smeets et al. 2009) complain of lower quality of life and are more prone to accidents or food poisoning. Parosmia-affected patients are also characterized by lower quality of life. GPA therapy should include counseling and support aimed at helping patients deal with olfactory dysfunction. Such as advice on nutrition, personal hygiene, and food or gas poisoning prevention (Blomqvist et al. 2004; Landis et al. 2003, 2009).

In the present study, screening of olfactory function was based on the commonly used olfactory testing method. The selection of a method is crucial for the results achieved, as well as for comparison with other studies. The method described in this study has also been adopted in a compact, screening version by Laudien et al. (2009) and Göktas et al. (2010). Sniffin' Sticks Test has been specifically designed for inhabitants of Central and Eastern Europe; the results have been standardized on a group of more than 3000 patients and are thus verifiably reliable. Unfortunately, modern olfactory diagnostics does not allow for a precise specification of a location of olfactory dysfunction. In contrast, standardized methods of trigeminal nerve assessment are limited; therefore this nerve was not separately tested in the present study.

In conclusion, active nasal and paranasal sinus inflammation in GPA leads to olfactory dysfunction, patients are often unaware of. The dysfunction is permanent and does not withdraw when the inflammatory process is under control. GPA therapy should include advice on nutrition, personal hygiene, and food poisoning prevention to help minimize adverse effects of olfactory dysfunction.

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

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Biochemistry of Oxidative Stress

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Abstract

Generation of reactive oxygen species is a physiological process that take place in every aerobic organism. Oxidative stress is defined as a disturbance in the balance between the production of free radicals and antioxidants in favor of the oxidants. The imbalance between those two fractions may potentially lead to cell damage at molecular level. Since oxidants are formed at a different rate as a normal product of aerobic metabolism, complex biochemical mechanisms are required to regulate the entire process.

Keywords

Antioxidation • Free radicals • Oxidative stress • Reactive oxygen species • Reactive nitrogen species

1 Introduction

Oxygen paradox is the term underscoring the fact that higher eukaryotic aerobic organisms cannot exist without oxygen, yet oxygen is inherently dangerous to their existence. This unique feature of oxygen relates directly to the fact that each oxygen atom has two unpaired electrons in separate orbitals in its outer shell, which makes it particularly prone to radical formation. In mitochondria, oxygen is reduced by the electron-transport chain to the water molecule. This is known as a complete reduction of oxygen. This relatively safe process has a serious flaw because the univalent reduction of oxygen generates reactive intermediates. Moreover, reductive environment of the cellular milieu may provide opportunities for oxygen to undergo an unexpected univalent partial reduction. Therefore, reactive oxygen species are common products in the aerobic environment and contribute to oxygen toxicity.

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2 Reactive Oxygen Species

Reactive oxygen species (ROS) are chemically reactive molecules (Table 1). All of ROS molecules manifest higher reactivity than triplet oxygen, which is an inorganic chemical in the basic state, with the chemical formula $O_2^{\bullet 2}$. Reactive forms are by-products of oxygen metabolism and play an important role in a variety of intracellular processes, like homeostasis and cell signaling (Fig. 1). Free radicals are created in redox reactions, radiolysis, photolysis, and homolytic fission where chemical bonds break and each of the newly created fragments preserves one of the originally-bounded electrons (Halliwell and Gutteridge 1984).

2.1 Sources of Free Radicals

Distinct mechanisms providing free radicals are based on the chain reaction. A sequence of reactions consists of three interrelated phases. The first step is initiated by superoxides, high temperature or UV-radiation and, in consequence, a free radical is formed. This process may involve the formation of free radicals from stable species or they may involve reactions of free radicals with stable species yielding distinct forms of free radicals. Additionally, a break of molecule's double bonds and ß-elimination, in which atomic bond breaks in ß-position, play a role in oxidative stress. When a sufficient amount of free radicals is produced, the next phase of the process begins, known as the prolongation, and the final product is synthetized. During the prolongation phase, free radicals act as accelerators and their total number remains stable. Finally, all reactions are attenuated, limiting the number of free radicals. Typically, it occurs when free radicals combine to form a more stable species (Morrison and Boyd 1992).

2.2 Mitochondrial Complex I

Free radicals and reactive oxygen species can be generated by external factors, such as ionizing radiation, UV-radiation, or ultrasounds. These biologically active molecules occur in mitochondria-the cellular power plants. While the main function of the mitochondria is production of ATP molecules, they also produce free radicals. In the respiratory chain, electrons from reduced substrates pass from protein Complexes I and II of the electron transport chain through Complexes III and IV to oxygen, forming water and causing protons to be pumped across the mitochondrial inner membrane. The passage of electrons through these complexes sustains the production of superoxide anion ($O_2^{\bullet -}$) and the formation of ATP. There are two major sideward reactions that accompany the main electron transfer pathway in mitochondria. Electrons may leak from the respiratory chain and react with oxygen to form superoxide, or the pumped protons may pass across the inner membranes, prohibiting ATP synthesis.

It is believed that Complex I (NADH/ubiquinone oxidoreductase) is responsible for the production of the highest amount of superoxide in healthy mammalian mitochondria, compared to

Table 1 Major reactive oxygen species (ROS)

Oxygen free radicals	Reactive nitrogen species (RNS)	Non-radical oxidants
$^1O_2$ (singlet oxygen)	$NO^{\bullet}$ (nitric oxide)	H_2O_2 (hydrogen peroxide)
$O_2^{\bullet -}$ (superoxide anion)	$NO_2^{\bullet}$ (nitric dioxide)	ONOOH (peroxynitrous acid)
$^{\bullet}OH$ (hydroxyl radical)		HClO (hypochlorous acid)
$HO_2^{\bullet}$ (hydroperoxyl radical)		HOSCN (hypothiocyanous acid)
Iron-oxygen complexes	Organic free radicals	Other
$Fe = O^{2+}$ (ferryl-oxygen complex)	$R–O^{\bullet}$ (alkoxy radical)	HIO (hypoiodous acid)
$Fe = O^{3+}$ (perferryl-oxygen complex)	$R\text{-}OO^{\bullet}$ (peroxide radical)	HClO (hypochlorous acid)
	$^{\bullet}QH$ (semiquinone radical)	
	$^{\bullet}QH^{-}$ (semiquinone anionradical)	HBrO (hypobromous acid)

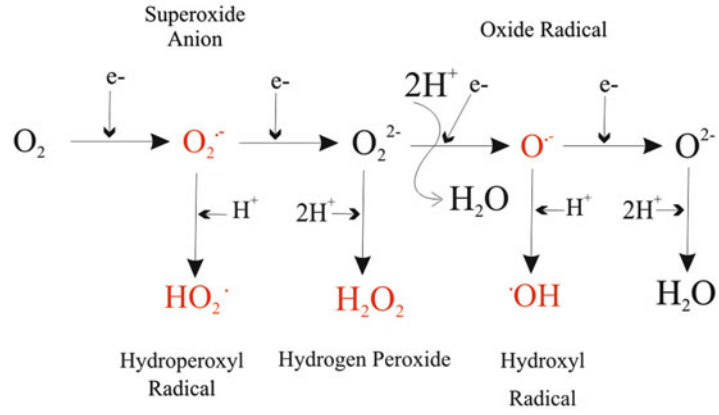

Fig. 1 Metabolism of oxygen and its by-products

other mitochondrial complexes. The main event which takes place in Complex I is oxidation of succinate, which then is used as a substrate for succinate dehydrogenase (Complex II). During production of oxidized succinate, the rate of superoxide generation is augmented within the cell. The possible mechanisms of $O_2^{\bullet-}$ production has been linked to the electron transfer chain. In the absence of NAD^+- linked substrates upon succinate oxidation in Complex II, the reverse electron transfer (RET) can spawn a fair amount of superoxide anion. The forward electron transfer is able to generate $O_2^{\bullet-}$ from the NAD^+- linked substrates at a much lower level than RET. As a consequence, when Complex I inhibitor rotenone is administered, the rate of superoxide generation is immediately decreased. Finally, high superoxide production from Complex I occurs during the reverse electron transport chain from succinate to NAD^+, which is driven by a high proton motive force generated from proton pumping by Complexes III and IV. This superoxide production occurs primarily on the matrix side of the inner membrane (Brand et al. 2004; Jezek and Hlavata 2005; Bleier and Drose 2013).

2.3 Mitochondrial Complex II

As mentioned above, the contribution of other complexes to the process of reactive oxygen species generation is low, but cannot be ignored. While Complex I is active only in physiological conditions, Complex II (succinate dehydrogenase) can produce superoxide anion ($O_2^{\bullet-}$), most probably by oxidation of the flavin semiquinone radical, in the absence of an immediate electron transfer partner. Some studies have shown that this reaction occurs *in vivo* only when Complex II is damaged by oxidative stress or aging. When Complex II is not altered, this event occurs only in hypoxic conditions. Low oxygen level switches Complex II activity from succinate dehydrogenase to the reverse electron transfer. As a result, succinate dehydrogenase starts to act as fumarate reductase and fumarate provides the terminal electron acceptor from the ubiquinol pool. As it was outlined above, this sequence of events contributes to very efficient $O_2^{\bullet-}$ production in the Complex I (Jezek and Hlavata 2005).

2.4 Mitochondrial Complex III

Mitochondrial cytochrome bc1 complex, also known as Complex III (ubiquinol/cytochrome c oxidoreductase), is also known as a producer of superoxide and derived reactive oxygen species within the mitochondrial respiratory chain. Complex III derived ROS have been linked to cellular redox signaling pathways, e.g., hypoxic stabilization of HIF-1α, but precise molecular mechanism governing this reaction remains largely unknown. It has been described how the

sequential oxidation and reduction of the lipid-loving electron carrier, Coenzyme Q10 (CoQ10), between the ubiquinol and ubiquinone forms, can result in the net pumping of protons across the inner mitochondrial membrane. Nowadays this pathway is considered as a blueprint of Complex III functionality (Mitchell 1976; Bleier and Drose 2013).

In short, in Complex III, the transfer of electrons from ubiquinol (UQH2) to cytochrome c (and the associated H^+ pumping) occurs within the Q cycle. UQH_2 diffuses from the membrane pool to its binding site called Qo (or Qp). This receptor is situated near the cytosolic surface of the inner mitochondrial membrane and co-localized with the iron-sulphur proteins (ISP). These proteins are also labeled as Rieske proteins and are components of cytochrome bc1 and cytochrome b6f complexes (Rieske et al. 1964; Jezek and Hlavata 2005). Initially, the electron is transferred directly from UQH_2 to the Rieske protein. Further, the electron is transported to cyt c1, and then to cyt c. The remaining proton is moved to the Qo site initiating the release of two H^+ to the cytosolic side and formation of $UQ^{\bullet-}$ at the Qo (Fig. 2). This event is only possible when transfer of the second electron to the low potential chain is retarded or blocked. Under standard conditions, $UQ^{\bullet-}$ is directly oxidized by cytochrome bL to UQ on the cytosolic side. Considering that O_2 is better soluble in lipids than water, and it can be found in a greater amount between lipid bilayer and aqueous intermembrane space, it is obvious that the O_2 will damage the mitochondrial membrane and reacts with $UQ^{\bullet-}$, especially when electron transport is slowed down by a high membrane potential. As a consequence of this reaction, superoxide ($O_2^{\bullet-}$) is generated. Moreover, due to the proximal location of the Qo site in the intermembrane space, the $O_2^{\bullet-}$, as an anion, should be rather moved from the membrane to the cytosolic side (Jezek and Hlavata 2005; Han et al. 2001).

2.5 NADPH Oxidase

The NADPH (nicotinamide adenine dinucleotide phosphate) oxidase of phagocytes is a multicomponent enzyme complex, containing two membrane and three cytosolic components (Babior et al. 2002). This enzymatic complex is crucial for phagocytes functioning, as respiratory burst consumes great amounts of reactive oxygen forms. This enzyme catalyses the conversion of oxygen to superoxide anion in the reaction: $NADPH + O_2 \rightarrow NADP^+ + H^+ + O_2^{\bullet-}$ (Fig. 3).

In further biochemical processes superoxide can be transformed to H_2O_2 which serves as a co-substrate for peroxidases, i.e., myeloperoxidase (MPO) in neutrophils and monocytes or eosinophil peroxidase. Classic heme peroxidase in neutrophils uses hydrogen

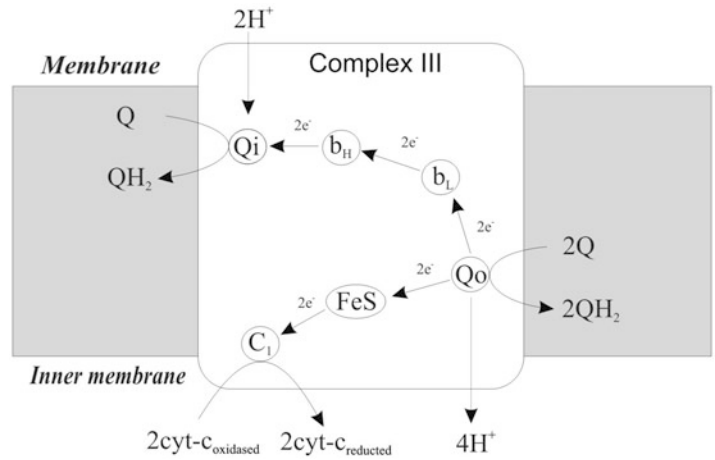

Fig. 2 The Q-cycle

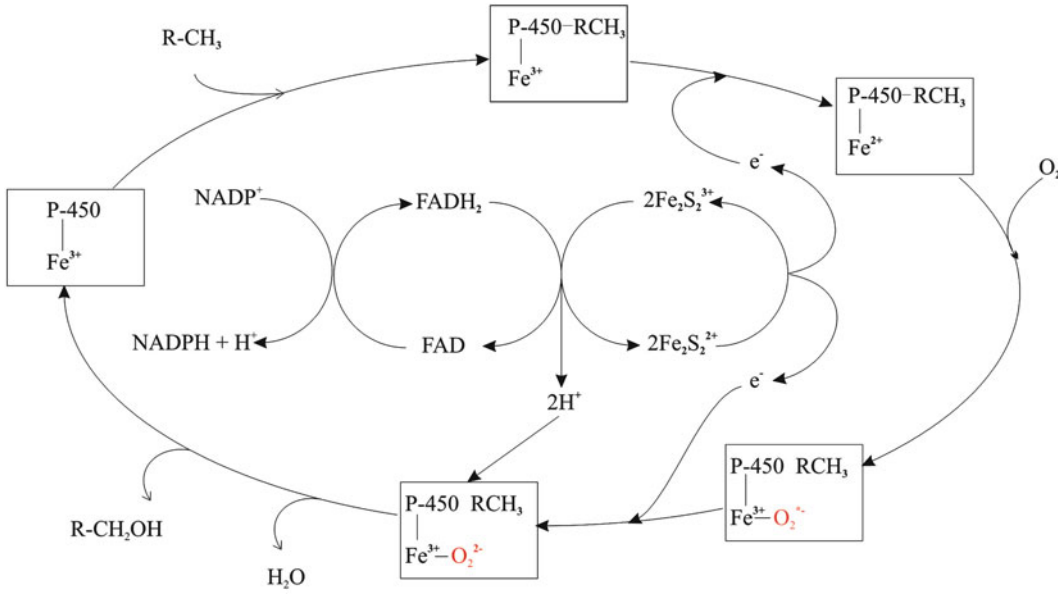

Fig. 3 Microsomal electron transport chain (Based on Paselk 2008)

peroxide to oxidize a multitude of aromatic chemical compounds by one-electron mechanism in order to receive substrate radicals. In very unique way MPO can oxidase chloride ions to the strong non-radical oxidant hypochlorous acid (HClO) (Hampton et al. 1998). When adequate levels of hydrogen peroxide and hypochlorous acid are produced, they react and produce singlet oxygen ($^1O_2$) which acts against double bonds in organic molecules (Babior et al. 2002).

NADPH oxidase can yield $O_2^{\bullet -}$, HClO, and hydroxyl radical. This reaction requires the presence of metal (M) ions and can be described as follows: $H_2O_2 + M^+ \rightarrow {}^{\bullet}OH + OH^- + M^{2+}$.

2.6 Microsomal Electron Transport Chain

This unique biochemical cascade of events is composed of Cytochrome P450 and NADPH-hemoprotein reductase. Flavin adenine dinucleotide and/or flavin mononucleotide serve as cofactors (Farrera and Fadeel 2013). Biotransformation of drugs and xenobiotic as well as production of bile acids and hormones are the main functions of this mitochondrial enzymatic chain. Ferryl- and perferryl-oxygen complexes are formed as by-products (Fig. 3).

2.7 Peroxisomes

Almost all eukaryotic cells are capable of producing H_2O_2. The main site of this ROS production chain is localized in peroxisomes. This bio-machinery engage several enzymatic complexes, a full list is compiled in Table 2 (Schrader and Fahimi 2004). These intracellular structures are potent source of H_2O_2 and can utilize this reactive oxygen form to synthetize biologically active compounds, like hydroxides. In the first step, H_2O_2 is decomposed by a tandem of enzymes, catalase and glutathione-peroxidase (GPx). In an alternative pathway, H_2O_2 is converted into hydroxyl radical. Subsequently, ${}^{\bullet}OH$ interacts with cell membrane and promotes peroxidation of unsaturated fatty acids. As a consequence, hydroxyperoxides are generated. They can be further broken down by the previously described tandem of peroxisomal enzymes. In both cases, superoxide anion ($O_2^{\bullet -}$) is released. This oxygen form can be utilized by another tandem of enzymes. In this event, manganese superoxide dismutase (MnSOD) and copper-zinc

Table 2 ROS generation in peroxisomes in mammals

Enzyme	Substrate	ROS
Acyl-CoA oxidase		H_2O_2
Palimtyl-CoA oxidase	Long chain fatty acids	
Pristanoyl-CoA oxidase	2-methyl branched-chain fatty acids	
Trihydroxycoprostanoyl –CoA oxidase	Bile acids intermediates	
D-Amino acid oxidase	D-Proline	H_2O_2
D-Asparate oxidase	D-Asparate, *N*-methyl-D-asparate	H_2O_2
Hydroxyacid oxidase	Glycolate, lactate	H_2O_2
Pipecolic acid oxidase	L-Pipecolic acid	H_2O_2
Polyamine oxidase	*N*-Acetyl spermine/spermidine	H_2O_2
Urate oxidase	Uric acid	H_2O_2
Xanthine oxidase	Xanthine	$O_2^{\bullet-}$
NO synthase	L-Arginine	$NO^{\bullet}$

Based on Schrader and Fahimi (2004)

superoxide dismutase react with $O_2^{\bullet-}$ and as a result H_2O_2 is formed. Alternatively, superoxide anion can be engaged in production of powerful oxidative compound known as peroxynitrite ($ONOO^-$). In order to perform this reaction, L-arginine must be transformed into $NO^{\bullet}$ in the process of oxidation in which nitric acid synthase is engaged (Takei et al. 1996). A schematic overview of peroxisomal enzymes and ROS generation is presented in Fig. 4.

2.8 Cholesterol Desmolase

Cholesterol desmolase, also known as cholesterol side-chain cleavage enzyme, is localized in the inner layer of mitochondria of steroidogenic tissues. This enzymatic complex is formed by three different proteins: cytochrome P450, adrenodoxin, and adrenodoxin reductase (mitochondrial flavoprotein with a FAD type coenzyme). The entire complex is responsible for converting cholesterol to pregnenolone. By-products of cholesterol hydroxylation react with oxygen to form $O_2^{\bullet-}$ anions (Takeshita et al. 1983; Lambeth and Kamin 1976).

2.9 Reactive Nitrogen Species

Mitochondria are potent ensembles able to produce reactive oxygen species (ROS) effectively, but they also generate reactive nitrogen species (RNS) and nitric radicals. This reaction is initiated by hypoxia. A limited availability of oxygen in a tissue augments the activity of mitochondrial nitric oxide synthases (mt-NOS). As a result, intracellular pool of nitric oxide (NO) increases. $NO^{\bullet}$ reacts with $O_2^{\bullet-}$ yielding peroxynitrite, a potent oxidising and nitrating agent ($NO^{\bullet} + O_2^{\bullet-} \rightarrow ONOO^-$). Peroxynitrite can react with a variety of biomolecules, such as glutathione, methionine, ascorbate, and nucleobases. It has also the ability to suppress the activity of mitochondrial Complexes I, II, and IV. In addition, it often initiates lipids peroxidation. Nitric dioxide can react with saturated fatty acids and proteins (Rom et al. 2013; Haynes et al. 2004).

2.10 Reactive Oxygen Species Homeostasis

The balance between ROS and RNS sources and antioxidant mechanisms is crucial for cell survival. This oxidative homeostasis differs between cells types and tissues. A disruption of this thin balance can be due either to enhanced or decreased antioxidative capacity of the cell and may be of serious physiological consequences.

When mitochondria are in their non-phosphorylating coupled state or when

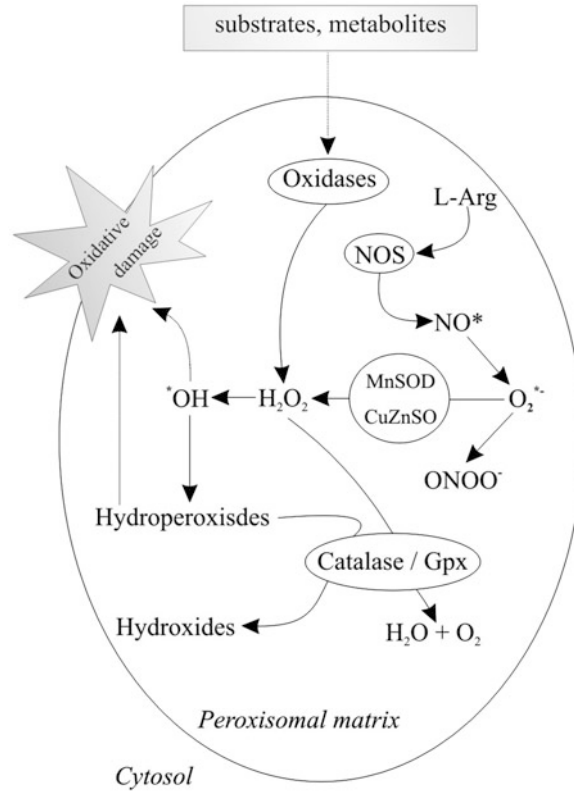

Fig. 4 Schematic overview of peroxisomal enzymes and ROS generation

reverse electron transport is active, substantial ROS production occurs. In other words, the mitochondrial ROS source is on. During this state mitochondria provide a substantial superoxide and H_2O_2 efflux to the cytosol. Elevated levels of cytosolic and extracellular ROS are balanced by the respective cytosolic and extracellular detoxification mechanisms. One of these unique mechanisms is the thioredoxin system, also called GSH/GSSH, other mechanisms can incorporate enzymatic complexes, like cytosolic and extracellular Cu-/Zn-SOD catalase. There are also non-enzymatic antioxidant systems in the cell. When overproduction of mitochondrial ROS occurs, the antioxidant defense system becomes overwhelmed and the balance is tilted toward oxidative stress.

In other conditions, when mitochondria are in their phosphorylated or uncoupled state, ROS production is diminished and the mitochondrial ROS source is almost switched off. In this situation, abundance of antioxidants occurs, which reduces the amount of ROS in the mitochondrial microenvironment; hence also in the cytosol and extracellular space. The antioxidants can even detoxify excreted products such as lipoproteins (Jezek and Hlavata 2005). The complexity of mechanisms underlying oxidative homeostasis is astonishing.

2.11 Reactive Oxygen Species and Biological Structures

ROS can be useful as a brick in intracellular signaling cascades or initiators of a range of processes. On the other hand, they can also target cell membrane and a lipid bilayer of organelles, where lipid peroxidation occurs. This process is a chain reaction affecting most often polyunsaturated fatty acids (PUFAs), as they contain multiple double bonds linked with methylene bridges ($–CH_2–$) that possess especially reactive hydrogens. Both polyunsaturated fatty acids, linoleates are considered to be the most abundant fraction of PUFA *in vivo*, and cholesterol are

oxidized by enzymatic and non-enzymatic mechanisms (Niki et al. 2005).

2.12 Non-enzymatic Lipid Peroxidation

The non-enzymatic process does not yield any specific products in contrast to enzymatic peroxidation. The entire process can be described as follows: $LH \rightarrow L^{\bullet} \rightarrow (O_2) \rightarrow LOO^{\bullet}$.

A complete free radical-mediated peroxidation of PUFAs incorporates five elementary reactions which influence each other. Firstly, hydrogen atom is transferred from PUFA to the chain initiating radical or chain carrying peroxyl radical to give a pentadienyl carbon-centered lipid radical. In the second process, a lipid radical reacts with molecular oxygen, therefore lipid peroxyl radical is generated. The third step is decomposition of the lipid peroxyl radical. As a result, oxygen and a lipid radical form. The next step engages rearrangement and cyclization of the peroxyl radical. The cyclization is important only for PUFAs having more than three double bonds, and it does not take place during the oxidation of linoleates. As it was mentioned above, this process is oxygen radical dependent. The cascade of these events can be initiated by: $^{\bullet}OH$ (hydroxyl radical), $HO_2^{\bullet}$ (hydroperoxyl radical), $NO^{\bullet}$ (nitric oxide), or $Fe = O^{3+}$ (perferryl-oxygen complex). Also non-enzymatic lipid oxidation products, i.e., lipophilic radicals: $L^{\bullet}$, $L–O^{\bullet}$, and $L–O–O^{\bullet}$ are expected to initiate additional peroxidation processes (Niki et al. 2005). As a consequence, there increases the level of lipophilic radicals and the following reactions occur:

$$L^{\bullet} + L^{\bullet} \rightarrow LL$$
$$LOO^{\bullet} + LOO^{\bullet} \rightarrow L = O + LOH + O_2$$
$$LOO^{\bullet} + L^{\bullet} \rightarrow L = O + LOH$$

2.13 Enzymatic Lipid Peroxidation

At the basic level enzymatic and non-enzymatic peroxidation do not differ much from each other. The mechanisms is almost the same, but due to the reason that certain enzymes catalyse peroxidation, distinct stereo- and region-specific products are formed during the enzymatic process. The main difference is that lipophilic radicals are transformed into anions. That process slows down the enzymatic production of superoxide, since lipophilic radicals are the main substrate for lipid peroxidizing enzymes, called lipoxygenases. The enzymatic pathway is responsible for production of a variety of specific biologically active molecules such as: prostaglandins, thromboxanes, leukotrienes and other arachidonic acid metabolites (Niki et al. 2005).

2.14 Influence of ROS on Proteins

Being highly active, ROS interact with virtually all cellular components modifying their properties. Modification of proteins induced by free radicals can result in the loss of their functions, but also can alter communication and incoming information from the environment due to the reason that living organisms use proteins for intra- and intercellular signaling pathways. At the very basic state, reactive oxygen species are able to cleave polypeptide chains and oxidize side chains of amino acids. Currently, at least four mechanisms of free radical induced peptide bond cleavage have been proposed: cleavage of alkoxyl derivative peptides *via* the α-amide pathway or the diamide pathway, oxidation of side chains of glutamyl and aspartyl residues, and finally oxidation of side chains of proline. In some cases, protein reversible modifications, such as S-glutathionylation, S-nitrosation, and methionine sulfoxidation may occur. However, formation of additional carbonyl groups on polypeptide chain (carbonylation) often takes place. This irreversible, non-enzymatic reaction is introduced by a variety of oxidative pathways.

Functional consequences of protein modification are clear-cut. Many a study shows that protein oxidation greatly influences the functional state of organisms and takes part in the aging process, responses to hyperoxia and hypoxia, or in heat shock proteins production, which makes the redox regulation a key player in different pathologies (Lushchak 2007). The main

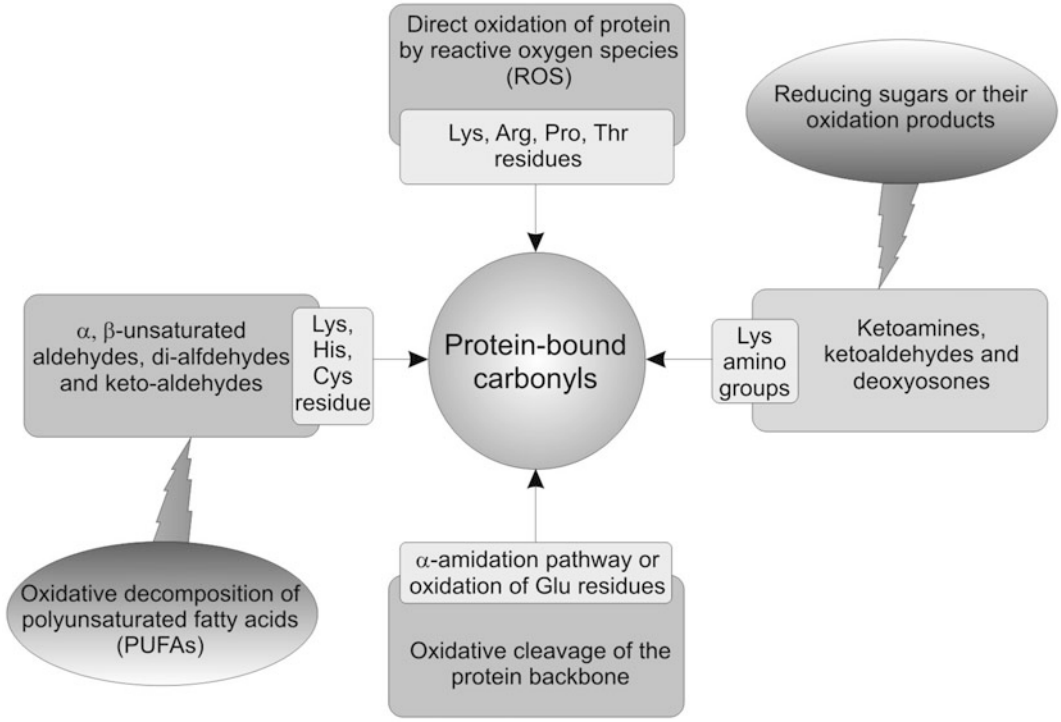

Fig. 5 The origin of carbonylated proteins (Based on Dalle-Donne et al. 2006)

pathways of protein carbonylation are presented in Fig. 5 (based on Dalle-Donne et al. 2006).

2.15 Influence of ROS on Carbohydrates and Nucleic Acids

Hydroxyl radicals react with carbohydrates by random removal of hydrogen atom from a carbohydrate, producing a carbon-cantered radical. This leads to chain breaks in molecules whose backbone is of carbohydrate origin. The entire process often involves intermediates such as peroxyl radicals.

Nucleic acids are more resistant to oxidation as compared to lipids and proteins, moreover any damage caused by ROS is rapidly repaired. Nevertheless, hydroxyl radicals can interact with double bonds of heterocyclic DNA bases and remove H^+ atom from the methyl group of thymine and from each of the C–H bonds of 2-deoxyribose. Further reactions of the then formed C- or N-centered radicals of DNA bases and C-centered radicals of sugar particles result in a variety of final products, like 8-hydroxy-2-deoxyguanosine (8-OHdG) or 5,6-dihydrouracil (5,6-diHUra) (Evans et al. 2004; Cooke et al. 2003).

3 Oxidative Stress

Constant interference of ROS with lipids, proteins, nucleic acids, and carbohydrates develops an array of free radicals, which can generate additional free radical, and influences crucial cell signaling pathways. When, cell or tissue is bombarded by enormous amounts of free radicals, oxidative stress occurs. This event is provoked by endogenous overproduction of free radicals, impaired cleaning system, or oxidative homeostasis dysfunction. Oxidative stress often brings several harmful consequences to a cell or even entire tissue. Main incidents are the following:

- Decrease level of ATP in a cell; free radicals efficiently inactivate glycolysis by inhibition

of glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$);
- Elevation of cytosolic Ca^{2+} *via* calcium pump inactivation;
- DNA damage by hydroxyl radicals or by calcium-dependent nuclease overactivation. A massive nucleic damage causes activation of NAD^+ ADP-ribosyltransferase and subsequently the cellular pool of NAD^+ and adenine nucleotides is diminished;
- Higher permeability of lipid bilayer caused by membrane depolarization induced by oxidative inhibition of potassium pump;
- Decrease of the intracellular level of glutathione and changes in proportions of reduced to oxidized form (GSH/GSSG).

As a final result of prolonged exposition to oxidative stress, the cell undergoes apoptosis or necrosis (Nakaya et al. 1992).

To prevent the damage done by oxidation of biological molecules a repertoire of factors serves as protective agents. These agents, when present at low concentrations compared with that of the substrate being oxidized, significantly delay or inhibit oxidation. They can be synthetized in the organism but also can be acquired from external sources. These compounds known as antioxidants show non-enzymatic as well as enzymatic nature and act in the opposite way to pro-oxidants. Non-enzymatic protection is based on two mechanisms. The first mechanism is deactivation of oxidative agents; thus, non-radical and non-reactive end-products are being formed. Secondly, non-enzymatic agents are involved in radicals transfer from crucial intracellular sites to less important compartments. This consists of transferring reactive species from hydrophobic sites to aqueous phase, i.e., from lipid layers to cytosol or from lipoproteins into the aqueous phase of plasma.

In addition to the non-enzymatic free radical clearance, eukaryotic organisms contain powerful antioxidant enzymes. The three major classes of antioxidant enzymes include the superoxide dismutases, catalases, and glutathione (GSH) peroxidases. These enzymes form a back-up antioxidant function. They catalyze the synthesis of GSH from glutathione disulphide (GSSG) by the flavoprotein GSSG reductase and they are able to transport and eliminate reactive compounds, e.g., the glutathione S-transferases and the transport system for the glutathione S-conjugates. Different subcellular sites and different cell types may contain varying amounts of the antioxidant enzymes (Sies 1997).

4 Conclusions

Cellular oxidative damage develops when the balance between the ROS-generating and ROS-scavenging systems favors the former. There is a thin line that separate those two mechanisms, and this line is often crossed. The number of diseases connected with oxidative stress and reactive oxygen species overproduction is astonishing. Despite our wide knowledge on many biological mechanisms involved in oxidative damage we still cannot efficiently stop unwanted oxidation. Until we discover new important facts about this phenomena, the best way to compensate biological oxidation is to increase external antioxidants intake.

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Usage of Over-the-Counter and Herbal Products in Common Cold in Poland: Findings from Consumer Survey

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Abstract

Upper respiratory tract infections are usually self-treated with synthetic and herbal over-the-counter products. The aim of the study was to assess the reasons for the purchase of those medications in Poland. We examined 413 adults, aged 18 and over (70.5 % of them were women) using a questionnaire. The findings demonstrate that oral synthetic products were used by 76 % of respondents, while herbal products by 30 %. Synthetic products were used mainly by educated people under 65 years of age, students, and the employed. Herbal products were used mainly by older people. In conclusion, synthetic products against common cold are perceived as more effective. Such medications are used by people who

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probably would like to recover and return to professional activity as quickly as possible. As they generally use more medications, they are at increased risk of adverse effects resulting from drug interactions, and they should be a target group for health education programs.

Keywords
Herbal products • Common cold • Consumer survey • Over-the-counter

1 Introduction

Common colds, which means viral upper respiratory tract infections, are generally mild diseases caused mainly by rhinoviruses, coronaviruses, influenza viruses, adenoviruses, and less often other viruses (human parainfluenza viruses, human respiratory syncytial virus, enteroviruses and metapneumovirus) (Johnston and Holgate 1996). Respiratory tract infections are particularly common among children. It is estimated that two to five colds might occur in adults, while children were prone to have seven to ten colds per year (Eccles 2005). The diagnosis of a common cold is easy and based on clinical symptoms, so that the majority of patients are usually self-diagnosed (Raal et al. 2013). Most upper respiratory tract infections are self-limiting and pose only a small risk of complications. The recommended management, therefore, typically involves self-care and relief of symptoms. The medications aim at bringing the ameliorating effects usually are anti-inflammatory and antipyretic drugs. Most of them are over-the-counter remedies, which are widely available (Ballengee and Turner 2014; ACPM 2011; Eccles 2006). The term 'over-the-counter' (OTC) means that the doctor's prescription is unneeded and it is possible to buy them anywhere, e.g., in a pharmacy, supermarket, at gas stations, or over the internet. To be registered as an OTC product, a drug must be safe in use for a predefined time (usually 3–5 days) in a mild clinical condition which can be self-diagnosed, e.g. common cold, gastrointestinal disorders, or pain (Woźniak-Holecka et al. 2012). The U.S. Food and Drug Administration and many other health care institutions have considered these drugs as safe and effective in the treatment of many common conditions (FDA 2014). The role of OTC medications in the healthcare system is rapidly increasing and such medications become even a more important group for therapy of the most common diseases worldwide (Ballengee and Turner 2014; Eccles 2006). It has been estimated that as many as 35 % of adults in the US use OTC products regularly (ACPM 2011). The non-pharmacy sales depend greatly on the existing legislation. The most recent list of substances admitted to trading outside pharmacies has been published in Poland in October 2010, and the list is trimmed compared with the previous ones. The use of OTC medications can bring many advantages, e.g., an easy and immediate access to therapy and less time-consuming visits to doctors. People become more responsible to care for their medical condition. The benefits also include a reduction of costs and a better organization of the entire health care system (ACPM 2011). OTC drugs usually have a higher therapeutic index than the prescribed ones and as a consequence they are less likely to cause side effects. However, it should be noticed that the use of OTC drugs could be potentially dangerous for health when the self-diagnosis is wrong or when drug interactions arise (ACPM 2011; WHO 2002). OTC products may also mask serious symptoms. The wide availability of the OTC medications and their intensive marketing promote an excessive use seen in some groups of people (e.g. adolescents and students – medical students particularly), lead to their misuse and addiction in some cases (Ford 2009). The OTC product

market and usage pattern are dictated by profit-orientated pharmaceutical companies, which leads to a situation in which consumers have unlimited access to a wide variety of pharmaceuticals. For these reasons, the use of OTC products must be subject to strict controls. The FDA supervises the drugs introduced to the market in the U.S. and the Compliance Policy Guide has removed a lot of medications from the market due to their unproven safety and efficacy (Fashner et al. 2012). It also should be emphasized that in some age groups of patients, OTC drugs are not recommended or even not allowed. For instance, FDA (2014) recommends the children under 2 years of age not be given any cold medications, while the American Academy of Pediatrics (AAP 1997) recommends to avoid such drugs up to 6 years of age. The role of doctors is to warn patients against excessive and reckless use of OTC drugs and to promote the awareness of benefits and untoward consequences of their overuse (Fashner et al. 2012).

Cold and flu products have been an important and increasing part of the Polish OTC market due to high flu and cold incidence rates. However, the attitude of Polish consumers toward the OTC market and demographic characteristics of people who use such medications have not yet been systematically studied. The aim of the present study was to assess the reasons which drive patients to use OTC medications against common cold. The essence of the research was to determine whether factors such as: education, sex, and place of residence influence the purchase of OTC drugs. We also attempted to evaluate the need for health education in the safe use of that class of drugs.

2 Methods

The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Bioethical Commission of the Medical University in Wroclaw (no KB-531/2013). The participants signed informed consent for the study form. They were recruited from December 2013 to April 2014. The main inclusion criterion was the age of at least 18 years. The study group consisted of 413 adults, the majority were women (291; 70.5 %), people between 18 and 24 years of age (171; 41.4 %), high school students (233; 56.4 %), inhabitants of towns with populations below 20,000 (188; 45.5 %). Detailed sociodemographic data are presented in Table 1.

Table 1 Sociodemographic data of patients

	n = 413	n	%
Gender	Women	291	70.5
	Men	80	19.4
Age	25 and below	171	41.4
	26–35	62	15.0
	36–45	94	22.8
	46–65	59	14.3
	Above 65	25	6.1
Place of residence	Town below 20,000	188	45.5
	20,000–100,000	116	28.1
	over 100,000	101	24.5
Education	Primary and vocational	34	8.2
	High school	233	56.4
	University	94	22.8

The study was of a self-reported survey type. The questionnaire consisted of 20 questions whose purpose was to estimate if Polish customers use OTC medications, herbal preparations, and other dietary supplements in the treatment of common cold. Questions referred to the place of purchase, frequency of purchase, the fact of informing or not a physician about using them together with other medications, and finally the source of information concerning the product. We also asked whether the leaflet accompanying the product package was sufficiently understandable and tackled the issue of side effects and possible health hazard.

The variables analyzed were qualitative (nominal) data. The χ^2 test was used to determine differences between variables. Depending on the size of 2×2 tables, the χ^2 test with Yates' correction, and also Fisher's exact test and correspondence analysis were used to determine the relationship between variables in Stub-and-Banner tables. The level of significance was defined as $p < 0.05$. A commercial Statistica

10.0 software by StatSoft (Tulsa, OK) was used for all data analyses.

3 Results

Oral synthetic OTC products against common cold were used by 76 % of respondents, while herbal products, mainly teas, were used by 30 % of them in the 12 months preceding the study; with some, rather minor overlap in the use of both products. The oral OTCs were used mainly by persons under 65 years of age ($\chi^2 = 36.6$, $df = 4$, $p < 0.001$) with secondary and higher education compared to those with primary and vocational education ($\chi^2 = 15.6$, $df = 2$, $p < 0.001$). These products were used more frequently by students and employed persons compared to unemployed and pensioners ($\chi^2 = 28.4$, $df = 3$, $p < 0.001$). The main place for purchasing these preparations was a pharmacy ($\chi^2 = 43.5$, $df = 1$, $p < 0.001$). The decision to choose a given preparation was primarily taken on the basis of its composition ($\chi^2 = 7.4$, $df = 1$, $p < 0.006$) and was a result of physician's recommendation ($\chi^2 = 9.9$, $df = 1$, $p < 0.002$).

Herbal teas were mainly used by persons over 35 years of age compared to younger persons ($\chi^2 = 27.3$, $df = 4$, $p = 0.001$). These formulations were mainly acquired from shops specialized in selling herbs ($\chi^2 = 48.3$, $df = 1$, $p < 0.001$). The persons who used them did not use any other synthetic oral preparations ($\chi^2 = 32.8$, $df = 1$, $p < 0.001$) and based their decision to get a given formulation on pharmacist's recommendation ($\chi^2 = 7.1$, $df = 1$, $p = 0.008$) and product's price ($\chi^2 = 13.3$, $df = 1$, $p = 0.0003$).

The information on the packaging or in leaflets accompanying medicinal preparations was more often read by women than men ($\chi^2 = 17.9$, $df = 1$, $p < 0.001$), by persons who were guided during the purchase by preparation's composition ($\chi^2 = 6.3$, $df = 1$, $p = 0.01$), and those who declared that the information was understandable and legible for them ($\chi^2 = 93.4$, $df = 1$, $p < 0.001$). Leaflets were more often read by persons with secondary and higher education than those with primary and vocational education ($\chi^2 = 16.4$, $df = 2$, $p < 0.0003$), those who declared that they got the information about the preparation from magazines ($\chi^2 = 10.5$, $df = 1$, $p < 0.001$), or those under the 65 years of age ($\chi^2 = 33.7$, $df = 4$, $p < 0.001$).

We found a significant relationship between the place of residence of the respondents and their education ($\chi^2 = 37.0$, $df = 10$, $p < 0.001$). Respondents with university education more often lived in big cities with more than 100,000 residents, persons with secondary education in cities of 50,000–80,000 inhabitants, and those with primary and vocational education lived in small towns (less than 20,000 residents). The relationship between the education of the respondents, using OTCs, and their age was also confirmed. Elderly persons over 65 years of age had mostly basic and vocational education, while younger persons aged 26–35 more often had university education ($\chi^2 = 143$, $df = 8$, $p < 0.0001$). In addition, there was a strong relationship between education and employment. The majority of the employed persons had university education, while the majority of the unemployed had basic or vocational education ($\chi^2 = 154.2$, $df = 6$, $p < 0.0001$).

The only statistically significant demographic variable which affected the use of OTC medications against common cold was professional activity. The employed persons (46.1 % of all respondents) and students used OTCs more frequently ($\chi^2 = 11.6$, $df = 3$, $p = 0.009$), and they were also more likely to take other medicines. Those who declared not taking OTCs also denied using other medicines ($\chi^2 = 9.02$, $df = 1$, $p = 0.003$).

We determined the effect of various factors on the use of OTCs against common cold:

- The price was important for people living in medium sized cities with the population from 50,000 to 500,000 ($\chi^2 = 12.7$, $df = 5$, $p = 0.026$) and for students, and to a lesser extent for the pensioners and the unemployed *vs.* the employed ($\chi^2 = 6.6$, $df = 2$, $p = 0.038$);

- The opinion of a physician was most important for the youngest persons below 25 years of age *vs.* persons from the oldest group of above 65 years of age and those aged 26–35 ($\chi^2 = 10.6$, df = 4, p = 0.031) and for persons with higher and secondary education *vs.* those with primary and vocational education ($\chi^2 = 6.6$, df = 2, p = 0.037);
- The opinion of friends was most important for persons living in medium-sized cities with a population of 50,000–80,000 ($\chi^2 = 17.7$, df = 5, p = 0.0034);
- The opinion of a pharmacist did not matter to people living in big and large cities with the population over 100,000 ($\chi^2 = 11.6$, df = 5, p = 0.041);
- The efficacy of a preparation was a more important criterion for people with university and secondary education than those with vocational and primary education ($\chi^2 = 26.9$, df = 2, p < 0.001) and more important for women than men ($\chi^2 = 8.9$, df = 1, p = 0.003).

4 Discussion

To our knowledge, this study has been the largest survey of consumers concerning the intake of OTC medications in Poland in recent years. Data on the self-medication in upper respiratory tract infections, including common colds, in Poland are limited and usually confined to aggregated commercial market data (EAS 2007).

The present study demonstrates that self-medication practices involving OTC and herbal products were widespread in the surveyed sample of the Polish population; the finding which has not yet been reported in the European surveys and reviews focused on factors that drive customers' behavior (Garcia-Alvarez et al. 2014; Bishop and Lewith 2010). Common cold and flu are predominantly self-diagnosed and self-treated with OTC medicines and herbal products (Mathens and Bellanger 2010; Eccles 2005). In the US, where data on the use of dietary supplements, including herbal supplements, have been collected more routinely, such supplements are used by about 18–19 % of the population (Wu et al. 2011), but available data range from 12 % in the Slone survey (Kaufman et al. (2002)) to 42 % in another national survey (Timbo et al. 2006). In a recent study conducted in Estonia, 67 % of respondents have had self-treated common cold and flu, using homemade remedies and medicinal plants followed by the combined use of herbal products and OTC medicines (Raal et al. 2013). These differences in the prevalence of OTC medicines across studies have been explained by the selection bias and differences in survey methodologies as well as by possible variations in health beliefs and health behavior of the different populations (Vargas-Murga et al. 2011; Schaffer et al. 2003). Likewise, we attribute higher figures obtained in the present study to the possible population bias. The majority of our subjects were frequent customers of pharmacies, which may not hold true for the general population.

Taking into account the aim of our study, we focused on determining the relationships between variables and correspondence analysis. Age and gender have been significant determinants of the consumption of OTC products and dietary supplements in general. Previous studies have shown a higher consumption among women than men (Menniti-Ippolito et al. 2002; National Center for Health Statistics 2002; Schaffer et al. 2003; Messerer et al. 2001; Nilsson et al. 2001; Nielsen et al. 2005) and also among old than young adults (Foote et al. 2003; Kelly et al. 2005; Bailey et al. 2013). In the present study we found a similar pattern of consumption; women bought more OTC products than men, and people >65 years of age more than younger adults. Likewise, people with higher education and being professionally active as well as students used OTC products more often than others. The influence of older age, as compared with the age of less than 35 years, on OTC consumption pattern was particularly visible concerning the use of herbal teas. This finding is consistent with the results of a survey in Estonia, where the general consumption of

medicinal plants increased with age (Raal et al. 2013). Older people have more time and usually suffer from chronic diseases, while younger people look for fast treatment options and prefer synthetic OTC medicines (Raal et al. 2013).

The present study indicates that consumers who use OTC products against common cold also use other OTC products. This is consistent with the finding from the U.S., where about half of the adult population admit using one or more dietary supplements (Bailey et al. 2013; Picciano et al. 2007). Analgesics and antipyretics are among the most commonly used self-medications in the U.S.; a national survey of 4263 adults conducted in 2002 estimated that more than 60 million people take such drugs at least several times a week (National Consumers League 2003). The OTC market and behavior of consumers in Poland seems quite similar to that in the U.S. In contrast, in the U.K. about 90 % of the consumers use only one supplement.

The results of the present survey should be considered in light of their limitations. The population sample encompassed mostly pharmacy consumers, who do not exactly represent the general Polish population. The retrospective nature of data collection might have introduced a data skew related to the retrieval of events or information from the past 12 months preceding the study.

5 Conclusions

The synthetic OTC products against common cold are perceived as more effective than herbal products. The OTCs are commonly used by the employed or students, who are busy and probably want to return to professional activity as quickly as possible. People using these preparations also often use more than one medicines. This group of people, being at increased risk of adverse effects resulting from polypharmacological interactions, should be a target group for the health education programs. From the marketing point of view, the most important target groups for products against common cold in Poland are women (using synthetic OTC medicines), and less educated and older people (using herbal products).

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RARβ Promoter Methylation as an Epigenetic Mechanism of Gene Silencing in Non-small Cell Lung Cancer

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Abstract

The retinoid acid receptor-β (*RARβ*) gene is one of the tumor suppressor genes (TSGs), which is frequently deleted or epigenetically silenced at an early stage of tumor progression. In this study we investigated the promoter methylation and expression status of the *RARβ* gene in 60 surgically resected non-small cell lung cancer (NSCLC) tissue samples and 60 corresponding unchanged lung tissue samples, using methylation-specific PCR and real-time-polymerase chain reaction (qPCR) techniques. We correlated the results with the pathological features of tumors and clinical characteristics of patients. qPCR analysis detected a significantly lower *RARβ* expression in the patients with adenocarcinoma (AC) and large cell carcinoma (LCC) than in those with squamous cell carcinoma (SCC) (AC *vs*. SCC, $p = 0.032$; AC and LCC *vs*. SCC, $p = 0.013$). Additionally, significantly lower expression of the *RARβ* gene was revealed in the patients with non-squamous cell cancer with a history of smoking assessed as pack-years (PY <40 *vs*. PY ≥ 40, $p = 0.045$). Regarding *RARβ* promoter methylation, we found significant differences in the methylation index in the SCC group when considering pTNM staging; with higher index values in T1a + T1b compared with T2a + T2b and T3 + T4 groups ($p = 0.024$). There was no correlation between the methylation status and expression level of the *RARβ* gene, which suggests that other molecular mechanisms influence the *RARβ* expression in NSCLC patients. In conclusion, different expression of the *RARβ* gene in SCC and NSCC makes the *RARβ* gene a valuable diagnostic marker for differentiating the NSCLC subtypes.

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Keywords
Gene expression • Lung cancer • NSCLC • Promoter methylation • Retinoid acid receptor-β gene • Smoking • Tumor suppressor gene

1 Introduction

Lung cancer is the most common malignancy in the world. It is one of the main causes of death among men between 50 and 70 years of age (Greenlee et al. 2000). On the basis of the histopathological classification, lung cancer is divided into two main groups: (1) small cell lung cancer (SCLC), representing about 20 % of cases, and (2) non-small cell lung cancer (NSCLC), which consists of approximately 75 % of all lung tumor diagnoses. Histologically, NSCLC is divided into the following subtypes – squamous cell carcinoma of lung (SCC), adenocarcinoma (AC) and large cell carcinoma (LCC) (Ettinger et al. 2010).

There are many documented risk factors associated with the development of lung cancer. Tobacco smoking is the cause of approximately 90 % of lung cancers among men and 80 % among women. There are many reports suggesting increased risk of developing lung cancer in relation to the number of smoked cigarettes and the duration of addiction (Agudo et al. 2012). For non-smokers it is assumed that simultaneous exposure to environmental tobacco smoke and genetic factors leads to the lung tumor development (Sasco et al. 2004). The studies show that accumulation of genetic abnormalities, such as the presence of genes polymorphisms, copy number variations, or epigenetic modifications, leads to the carcinogenesis in lung tissue (Word et al. 2013; Souto-Garcia et al. 2012; Staaf et al. 2013). Among the epigenetic mechanisms, the most important is methylation of promoter regions of the tumor suppressor genes (TSGs), causing their functional silencing.

The gene encoding the retinoic acid receptor beta (*RARβ*) is a tumor suppressor gene, located in chromosomal region 3p24.2. Retinoic acid receptors have α, β, and γ subclasses which reveal different levels of expression during human development and in adulthood. It is believed that *RARβ* plays a key role in the regulation of TSGs, epithelial cell growth, and it is downregulated in many human tumors (Gao et al. 2013; Sirchia et al. 2002). There are many reports suggesting a fundamental role of *RARβ* promoter methylation in increased risk of many human cancers, including lung cancer (Gao et al. 2013; Brabender et al. 2005; Sirchia et al. 2002; Virmani et al. 2000). The aim of the present study was to evaluate promoter methylation status of the *RARβ* gene in NSCLC patients.

2 Methods

The procedures used in the study were approved by the Ethics Committee of the Medical University of Lodz, Poland and written informed consent was received from each patient. There were 60 patients with confirmed primary NSCLCs (24 women, mean age 63.1 ± 7.8 years and 36 men, mean age 65.8 ± 7.3 years) enrolled into the study. The patients were untreated with chemo- or radiotherapy. This study was a branch of a greater project devoted to the investigation of the role of tumor suppressor genes in the mortality of lung cancer. The study was performed on lung tissue samples taken from the same patients as reported in a previous study that has highlighted the importance of the *FAM107A* gene in lung carcinogenesis (Pastuszak-Lewandoska et al. 2015).

Concerning the smoking history, five patients were non-smokers and 55 were smokers. Of the smokers, 32 were current smokers and 23 were ex-smokers. Among the smokers, regardless of the current smoking status, there were 26 patients who smoked for less than 40 years and 29 patients who smoked for more than 40 years.

Additionally, patients were divided into subgroups according to the number of cigarettes smoked, expressed in pack-years (PY), calculated according to the NCI Dictionary of Cancer Terms (1 pack year is equal to 20 cigarettes smoked per day for 1 year). Among the patients smoking up to 40 PY the following histopathological types of NSCLC were recognized: SCC (n = 14), AC (n = 13), and LCC (n = 4). In those smoking ≥40 PY the histopathological types were the following: SCC (n = 20), AC (n = 8), and LCC (n = 1).

2.1 Characterization of the NSCLC Tissue Samples

Lung tissue samples (100–150 mg) were obtained from patients with preoperatively diagnosed lung cancer who had undergone pulmonectomy or lobectomy at the Department of Thoracic Surgery, General and Oncologic Surgery, Medical University of Lodz, Poland, during the period of July 2010–June 2013. Tissue samples, immediately after resection, were collected in RNAlater® buffer (Qiagen; Hilden, Germany). Additionally, the adjacent non-cancerous macroscopically unchanged tissue (100 mg; 10 cm distant from the primary lesion) was resected from the same patient as control (C). Each tissue sample was divided into smaller parts (30–50 mg) for individual analysis. All samples were frozen at −80 °C.

The resected specimens were post-operatively histopathologically evaluated and classified according to the American Joint Committee on Cancer Staging (AJCC 2010; Goldstraw et al. 2007) and post-operative Tumor Node Metastasis classification (Goldstraw et al. 2007). Histopathological assessments of tumor specimens were obtained from pathomorphologic reports, and were as follows: squamous cell carcinoma (SCC, n = 34), adenocarcinoma (AC, n = 21), and large cell carcinoma (LCC, n = 5). The results of histopathological verification of tumor specimens, based on pathomorphologic reports, are summarized in Table 1.

Table 1 Histopathologic verifications of NSCLC samples

Histopathologic type of NSCLC	
Squamous cell carcinoma (SCC)	34 (57 %)
Non-squamous cell carcinoma (NSCC)	26 (43 %)
Adenocarcinoma (AC)	21 (35 %)
Large cell carcinoma (LCC)	5 (8 %)
AJCC	
IA/IB	12 (20 %)
IIA/IIB	21 (35 %)
III A/IIIB	27 (45 %)
pTNM	
T1	12 (20 %)
T2	33 (55 %)
T3-4	15 (25 %)

NSCLC non-small cell lung cancer, *AJCC* American Joint Committee on Cancer Staging, *pTNM* post-operative Tumor Node Metastasis classification

2.2 RNA Extraction and Expression Analysis of *RARβ*

Total RNA was extracted from tissue samples using Universal RNA Purification Kit (Eurx; Gdansk, Poland) according to the manufacturer's recommendations. The qualitative and quantitative assessments of RNA samples were determined by minielectrophoresis in polyacrylamide gel, using RNA 6000 Nano LabChip kit (Agilent 2100 Bioanalyzer; Santa Clara, CA)

Complementary DNA was transcribed from 1000 ng of total RNA, using a high-capacity cDNA reverse transcription kit (Applied Biosystems; Carlsbad, CA) in a total volume of 20 μl per reaction. Reverse transcription (RT) master mix contained: 10x RT buffer, 25x dNTP mix (100 mM), 10x RT random primers, MultiScribeTM reverse transcriptase, RNase inhibitor and nuclease-free water. RT reaction were conducted in a thermocycler (SureCycler 8800; Agilent Technologies, Santa Clara, CA) in the following conditions: 10 min at 25 °C, followed by 120 min at 37 °C, then the samples were heated to 85 °C for 5 s, and hold at 4 °C.

The relative expression of *RARβ* was assessed in qPCR reactions in ABI PRISM 7900HT real-time PCR system, using micro fluidic cards (Applied Biosystems; Carlsbad, CA) with

pre-loaded selected assays: Hs00977140_m1 for the *RARβ* (retinoid acid receptor beta) gene and Hs00382667_m1 for ESD (esterase D) as the reference gene.

The relative expression of the studied samples was assessed using the comparative $\Delta\Delta C_T$ method (TaqMan Relative Quantification Assay software; Applied Biosystems, Carlsbad, CA) and presented as relative expression (RQ) value, adjusted to ESD (endogenous control) expression level. RNA isolated from normal lung tissue (Human Lung Total RNA, Ambion; Life Technologies, Carlsbad, CA) served as calibrator sample for which RQ = 1. RNAs obtained from macroscopically unchanged lung tissues served as a control group.

Each sample-specific PCR mix contained 50 µl cDNA (50 ng) and 50 µl TaqMan universal master mix (Applied Biosystems, Carlsbad, CA). TaqMan Array card was centrifuged twice for 1 min at 1200 rpm to fill the wells with PCR mixture. Then, it was sealed with a TaqMan array micro fluidic card sealer and placed in the Applied Biosystems 7900HT fast real-time PCR system (Applied Biosystems; Carlsbad, CA). The PCR conditions were as follows: after initial incubation at 50 °C for 2 min and AmpliTaq Gold® DNA polymerase activation at 94.5 °C for 10 min, real-time PCR amplification was processed in 40 cycles of 30 s, and denaturation step at 97 °C, followed by 1 min elongation step at 59.7 °C.

2.3 DNA Isolation and Promoter Methylation Analysis of *RARβ*

The isolation of genomic DNA from NSCLC specimens and the matching macroscopically unchanged lung tissue samples were performed using a QIAamp DNA mini kit (Qiagen; Hilden, Germany), according to the manufacturer's protocol. The quality and quantity of isolated DNA was spectrophotometrically assessed, by measuring the absorbance at a wavelength of 260/280 nm using Eppendorf BioPhotometrTM Plus (Eppendorf; Hamburg, Germany). DNA samples with a 260/280 nm ratio in the range 1.8–2.0 were considered as high quality and used for further analysis.

Methylation status of the *RARβ* gene was assessed by methylation-specific polymerase chain reaction (MSP) using bisulfite converted DNA. Genomic DNA (1 µg) was modified with sodium bisulfite, using the CpGenomeTM turbo bisulfide modification kit (Chemicon International; Millipore, Temecula, CA), according to the manufacturer's protocol. The conventional MSP was performed in duplicate for each sodium bisulfite modified DNA sample, using AmpliTaq Gold® 360 DNA polymerase (Applied Biosystems; Carlsbad, CA). Amplifications were conducted in a total volume of 12.5 µl in a thermocycler (SureCycler 8800; Agilent Technologies; Santa Clara, CA). MSP master mix contained: 1000 ng DNA, 0.7 µM of each primer, 2.5 µM dNTPs mix, 2.5 µM $MgCl_2$, hot start AmpliTaq Gold 360 Polymerase (5U/µl), 10x universal PCR buffer and nuclease-free water.

The set of primers for the *RARβ* gene was flanking the 1 kb 5′ region upstream from the translation start point. The primers for methylation-specific PCR were designed according to the criteria described by Feltus et al. (2003). The sequences of primers (forward F and reverse R) methylated (M) and unmethylated (U) were as follows: GTGTTTAACGTGAGTTAGGAGTAGC (MF), ACAACCAAAAAAACAAACAAC (MR), TGGTGTTTAATGTGAGTTAGGAGTAGT (UF), ACAACCAAAAAAACAAACAACAAA (UR). PCR conditions were: 95 °C for 5 min (initial denaturation), followed by 40 cycles: 95 °C for 45 s (denaturation), 57.5 °C for 45 s (annealing), 72 °C for 1 min (elongation), and 72 °C for 10 min (final elongation).

In each PCR reaction, positive and negative MSP controls were included. CpGenome universal methylated DNA (enzymatically methylated human male genomic DNA) served as a positive methylation control and CpGenome universal unmethylated DNA (human fetal cell line) was used as a negative control (Chemicon International Millipore; Temecula, CA). Additionally, blank samples with nuclease-free water were used instead of DNA as a control for PCR contamination.

The MSP products were electrophoretically separated on 2 % agarose gel and concentrations (ng) of MSP products (U and M DNA alleles) were spectrophotometrically quantified (ng/μl), using DNA1000 LabChip kit, on a bioanalyzer (2100 Bioanalyzer; Agilent Technologies, Santa Clara, CA). Afterwards, the methylation indexes (MI) were assessed for each sample. The averaged MI, based on three repeats of amplification reaction, were calculated according to the formula MI = (M)/(M + U), where (M) stands for the methylated and (U) for unmethylated allele concentration, respectively.

2.4 Statistical Analysis

Differences in the value of relative expression (RQ) and in the methylation level (MI) of *RARβ* between NSCLC subtypes (SCC, AC, and LSCC) and between tumor tissue (NSCLC) and matching macroscopically unchanged tissue (control group) were assessed with the Kruskal-Wallis and Mann-Whitney U tests. Spearman's rank correlation coefficient was used to evaluate the relationship between the expression level (RQ values) or the methylation status of the *RARβ* gene and the examined patients' characteristics: age, gender, history of smoking, and tumor staging. The results of RQ values were presented as means ± SEM and means ± SD values. The level of statistical significance was set at $p < 0.05$. Statistica for Windows 10.0 program (StatSoft, Cracow, Poland) was applied for calculations.

3 Results

3.1 *RARβ* Gene Expression

In the histopathological NSCLC subtypes, decreased expression of the *RARβ* gene (RQ value <1) was observed in 50 % of the 60 samples studied. The range of decreased frequency was 38–80 %, depending on the histotype. Decreased expression of *RARβ* was most frequently observed in LCC (80 %), followed by AC (62 %), and by SCC (38 %). Regarding the macroscopically unchanged lung tissue, the mean RQ value was 1.28. The results are presented in Table 2. We did not find significant differences in the expression level of *RARβ* between cancerous tissue and adjacent macroscopically unchanged tissue obtained from the same patient.

There were significant differences in RQ values of the *RARβ* gene between all studied histopathological subtypes (SCC, AC, and LCC) ($p = 0.043$). A significant difference was observed between the SCC and NSCC groups (Fig. 1); the latter group consisted of AC and LCC subtypes. Likewise, significantly lower RQ values were observed in the AC group when we compared with SCC (Fig. 2).

There were no significant correlations between the RQ values of *RARβ* and the clinical features of the NSCLC patients, i.e., patients' age (under 60, 61–70, and over 70 years of age; $p = 0.617$), gender ($p = 0.476$), and history of smoking (assessed as PY: $p = 0.085$ and

Table 2 *RARβ* expression levels (RQ values) assessed by the $\Delta\Delta C_T$ method and percentage of samples with decreased and non-decreased expression compared with the calibrator in all studied histopathological NSCLC subtypes

			Number (%) of samples with:	
Histopathological NSCLC subtype	Median	Mean RQ (range)	RQ value <1	RQ value >1
SCC (n = 34)	1.49	1.65 (0.20–4.90)	13 (38 %)	21 (62 %)
AC (n = 21)	0.87	1.30 (0.29–9.27)	13 (62 %)	8 (38 %)
LCC (n = 5)	0.82	0.80 (0.33–1.23)	4 (80 %)	1 (20 %)
Total (n = 60)	1.07	1.46 (0.20–9.27)	30 (50 %)	30 (50 %)

NSCLC non-small cell lung cancer, *SCC* squamous cell carcinoma, *AC* adenocarcinoma, *LCC* large cell carcinoma

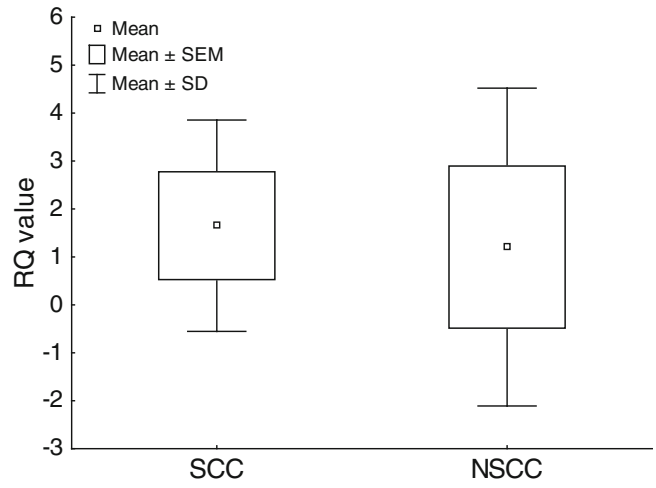

Fig. 1 Expression of the *RARβ* gene in squamous cell carcinoma (SCC) and in the non-small cell lung cancer (NSCC) group which included AC and LCC subtypes (box-and-whisker plot; p = 0.013; Mann-Whitney U test)

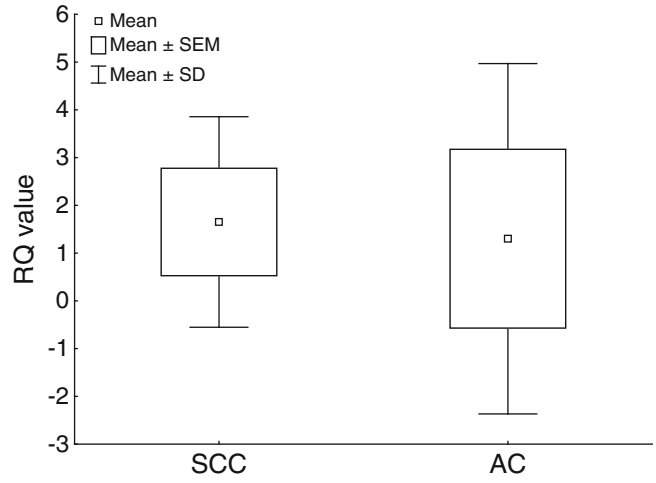

Fig. 2 Expression of the *RARβ* gene in squamous cell carcinoma (SCC) and adenocarcinoma (AC) (box-and-whisker plot; p = 0.032; Mann-Whitney U test)

cigarette addiction in a lifetime: p = 0.621). Interestingly, in the NSCC patients, significantly lower expression of the *RARβ* gene was revealed in the group with a history of smoking assessed as PY (PY < 40 *vs.* PY ≥ 40, p = 0.045). The analysis did not reveal any associations between *RARβ* expression and pTNM (p = 0.821) or AJCC (p = 0.929) classification.

3.2 Methylation Status of *RARβ* Gene

The presence of both methylated alleles (MI value = 1) was found in 19 % of NSCLC samples, while 65 % specimens had both methylated (M) and unmethylated (U) alleles (Table 3). There were no significant differences in MI values for the *RARβ* gene among the NSCLC histopathological subtypes SCC, AC, and LCC (p = 0.712). Nor were there differences in MI values regarding the patients' gender (p = 0.911), age (under 60, 61–70, and over 70 years; p = 0.952), or history of smoking (assessed as PY: p = 0.913 and cigarette addiction in a lifetime: p = 0.472).

Likewise, there were no significant differences between MI values and tumor size, according to pTNM (p = 0.420) or AJCC classification (p = 0.158). However, differences were found in relation to the tumor size classified

Table 3 Methylation status, expressed as MI (mean, range) in the studied histopathological NSCLC subtypes

		Number (%) of samples with:		
Histopathological NSCLC subtype	MI mean value (range)	MI value = 1 (both M alleles)	MI value =0 (both U alleles)	MI value >0 and <1 (M + U alleles)
SCC (n = 34)	0.51 (0–1)	6 (18 %)	3 (9 %)	25 (73 %)
AC (n = 19)	0.43 (0–1)	4 (21 %)	5 (26 %)	10 (53 %)
LCC (n = 5)	0.48 (0–1)	1 (20 %)	1 (20 %)	3 (60 %)
Total (n = 58)	0.48 (0–1)	11 (19 %)	9 (16 %)	38 (65 %)

NSCC non-small cell lung cancer, *MI* methylation index: MI = (M)/(M + U), *M* methylated, *U* unmethylated

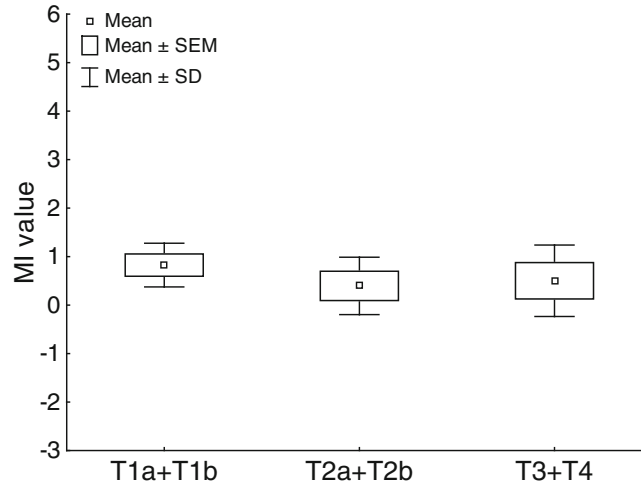

Fig. 3 Methylation of the *RARβ* gene in squamous cell carcinoma (SCC) classified according to pTNM staging in relation to tumor size (box-and-whisker plot; p = 0.024; Kruskal-Wallis test)

according to pTNM staging in the SCC group as presented in Fig. 3. A significantly higher MI value of *RARβ* was present in the T1a + T1b group as compared with the T2a + T2b or T3 + T4 group (p = 0.024; Kruskal–Wallis test)

There were no significant associations between the RQ or MI values of the *RARβ* gene and the patients' age; gender, TNM and AJCC classifications, histopathological subtypes, and smoking history in the whole NSCLC group.

4 Discussion

Due to a high prevalence and mortality, lung cancer is the subject of many studies. The identification of lung cancer genetic and epigenetic markers with diagnostic or prognostic values becomes an important tool for future molecular oncology and personalized therapy. Promoter methylation of TSGs plays an increasingly valuable role in carcinogenesis, including NSCLC. Among many TSGs claimed as being of importance in lung cancer development, the *RARβ* gene encoding the retinoic acid receptor (RARβ) is frequently deleted in different types of lung tumors. Since retinoic acid is essential for normal epithelial cell growth, regulation and cell differentiation, the molecular defect of *RARβ* might be expected to contribute to oncogenesis.

The *RARβ* gene was chosen for this study because of its critical location for lung carcinogenesis on the short arm of chromosome 3 (3p) which is recognized as a 'hot spot mutation site' in lung cancer. There are many reports regarding a reduced *RARβ* gene expression in tumorigenesis. It has been recognized that the effect of *RARβ* tumor suppressor gene repression increases the risk of development of several cancers, including prostate (Gao et al. 2013),

breast (Sirchia et al. 2002), ovary (Bhagat et al. 2014), bladder (Eissa et al. 2012), and also lung cancer (Brabender et al. 2005; Virmani et al. 2000; Picard et al. 1999).

In the present study we analyzed the epigenetic regulation, i.e., expression level and promoter methylation of the *RARβ* gene, in NSCLC patients. We also assessed the relationship between the clinical features of patients, histopathological subtypes of lung tumors and epigenetic *RARβ* regulation. To the best of our knowledge, the study focused on these epigenetic mechanisms is the first one conducted in the Polish population. We observed a *RARβ* gene downregulation in 50 % of NSCLC samples. The frequency of decreased *RARβ* expression was in a range of 38–80 %, depending on the NSCLC histotype, suggesting high genetic heterogeneity of tumor tissue cells. A decrease in *RARβ* expression was most frequently detected in LCC (80 %), and it was the least frequent in the SCC (38 %) subtype. These findings are similar to those obtained in other studies in which the *RARβ* gene expression was reduced to the level of 59 % or 63 % (Brabender et al. 2005; Picard et al. 1999). The results concerning the expression of *RARβ* in tumor tissue compared with the matching normal tissue in the studies cited above have been statistically significant, but the authors do not report the frequency of *RARβ* expression in particular NSCLC histotypes. Although we failed to find such an overall significance, we demonstrate a significant difference in *RARβ* gene expression in SCC compared with NSCC.

Regarding the NSCLC tumor staging and cigarette smoking, we did not find any association with *RARβ* expression. This is in accordance with the results of other author (Brabender et al. 2005; Picard et al. 1999). However, we documented significant differences in *RARβ* expression depending on the history of smoking as assessed as PY in the NSCC group, i.e., the expression was significantly higher in the group PY $\geq$40. The amount of cigarettes smoked over a lifetime seems to underscore the increase in *RARβ* expression in NSCC. This finding confirms the results of others (Liu et al. 2008) who show that cigarette smoking induces epigenomic alterations leading to lung carcinogenesis.

The interesting results concerning a low *RARβ* gene expression and tumor stage have been obtained by Brabender et al. (2005), who demonstrate a correlation between stage I and stage IIIa NSCLC ($p < 0.001$). The results suggest an association between low *RARβ* gene expression and worse prognosis in NSCLC patients.

The expression pattern of tumor suppressor genes depends on several genetic mechanisms that account for the availability of gene transcription. The loss of heterozygosity (LOH), microsatellite instability (MSI), the existence of polymorphisms, and epigenetic mechanisms underlie a disrupted expression of TSGs in lung carcinogenesis (Word et al. 2013; Staaf et al. 2013; Souto-Garcia et al. 2012). Among epigenetic mechanisms, apart from histone modifications and miRNA regulation, the most important is methylation of promoter regions of TSGs, causing functional silencing of those genes. To date, there are several reports concerning promoter methylation of *RARβ* gene. The results of these studies partly correspond with our findings. We found the presence of at least one methylated allele of *RARβ* gene in 84.5 % NSCLC samples, while both methylated alleles – indicating a 100 % status of *RARβ* gene – were present in 19 % specimens. The results of other authors are ambiguous and do not provide the methylation index, reporting only the frequency of methylated *RARβ* allele. This frequency ranges from 80 % (Li et al. 2012), which is similar to our present result, through 50–58 % (Liu et al. 2008; Tan et al. 2012), which grossly corresponds to our finding of 65 % (indicating the presence of one methylated and the other unmethylated *RARβ* allele), and to 30 % (Scesnaite et al. 2012; Zhang et al. 2011), which is much lower than in the percentage noted in the present study.

The evaluation of *RARβ* methylation status as an early predictor, dependening on the stage of a tumor, and differentiating marker, dependening on the histological subtype of NSCLC, also are ambiguous. In the present study, differences between the values of *RARβ* methylation and clinicopathological features (tumor staging or histopathological type) were insignificant. To

this end, our results are in conformity with those of other authors (Li et al. 2012; Scesnaite et al. 2012; Liu et al. 2008). However, in some reports a higher prevalence of *RARβ* promoter methylation has been found in patients with advanced stage tumors – TNM III/IV than in those with TNM I (Tan et al. 2012; Zhang et al. 2011). On the other hand, methylation status of several genes, including *RARβ,* allowed differentiating stage I NSCLC from non-cancerous lung diseases with a high sensitivity and specificity (Zhao et al. 2013). In the present study, we demonstrate significant differences in *RARβ* methylation depending on the tumors size in SCC, i.e., the methylation was higher in T1 group. The corollary is that hypermethylation of the *RARβ* gene has important implications in the pathogenesis of SCC; a histotype being strictly associated with cigarette smoke exposure. Indeed, an *in vitro* study on the exposure of respiratory epithelium to cigarette smoke indicates a role of early epigenetic mechanisms, regulating gene expression, in pulmonary carcinogenesis (Liu et al. 2010).

In the present study we did not find any significant associations between *RARβ* promoter methylation and tumor histology, which is in line with other reports (Li et al. 2012; Zhang et al. 2011; Liu et al. 2008). However, there are some findings that *RARβ* methylation is less frequently found in AC than SCC, but in co-methylation with *SIX6* and *SOX1* genes (Zhao et al. 2013). Apart from that, Scesnaite et al. (2012) have reported that *RARβ* methylation is significantly less frequent in other than AC histologic lung subtypes. In the present study, the presence of methylated *RARβ* allele was found in nearly 74 % of AC specimens.

As far as gender and *RARβ* methylation status are concerned, we did not find any mutual association. That is contrast to Tan et al. (2012) who have found a higher *RARβ* methylation frequency in men; however, Scesnaite et al. (2012) have reported the opposite results. Likewise, regarding the smoking history, we did not find any significant correlation between cigarette smoking and *RARβ* methylation status. It is in accordance with some authors (Scesnaite et al. 2012), although not confirmed by others who demonstrated a higher degree of *RARβ* methylation in smokers compared with nonsmokers with NSCLC (Tan et al. 2012).

The present study indicates a large discrepancy and ambiguity of the results concerning the effect of *RARβ* promoter methylation in NSCLC development. The differences between the results obtained in this study and those of other authors may result from different groups of patients, ethnic variability, or methods used. The involvement of other molecular events such as genomic instability, gene polymorphisms, or other epigenetic mechanisms, also significantly affect *RARβ* gene expression in NSCLC development. Recent studies show the possibility of indirect effects of methylation of miRNA genes (miR-9-1, miR-9-3, miR-34b/c, and miR-193a) on the level of expression of the *RARβ* gene and the regulation of retinoid pathway in lung cancer development (Nervi and Grignani 2014; Khodyrev et al. 2012).

In conclusion, the present study demonstrates a value of *RARβ* gene expression as a marker differentiating subtypes of NSCLC. The lack of correlation between the expression level of *RARβ* and the promoter methylation status of this gene suggests a role of yet another molecular mechanisms in *RARβ* downregulation in NSCLC. Further delineation of the mechanisms of action and resistance of retinoids in lung cancer is required to achieve clear benefits for patients.

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Conflicts of Interest The authors have no conflicts of interest concerning this study.

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Cardiovascular Risk Factors in Obese Children and Adolescents

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Abstract

The aim of the study was to analyze cardiometabolic risk factors and carotid intima-media thickness (IMT) in obese children. We studied 122 obese children fulfilling the criteria of the International Obesity Task Force and 58 non-obese children. Anthropometric parameters, blood pressure, lipid profile, C-reactive protein, and adiponectin were assessed in all children. Glucose and insulin during the oral glucose tolerance test were assessed in obese children. The IMT was determined using ultrasound B-mode imaging in 81 obese and 32 non-obese children. We found that obese children had significantly higher levels of lipid and other non-lipid atherogenic indicators, but lower levels of adiponectin compared with non-obese children. The difference in the mean carotid IMT was insignificant in the two groups. Taking the combined groups, the level of adiponectin correlated negatively with body mass index and lipid atherogenic indicators. The IMT strongly correlated with systolic blood pressure in obese children. In the children fulfilling the criteria of metabolic syndrome, 17 out of the 84 obese children older than 10 years of age, IMT was greater than in those who did not fulfil these criteria. We conclude that the coexistence of abdominal obesity with abnormal lipid profile and hypertension leads to the early development of atherosclerosis accompanied by increased carotid intima-media thickness. Obesity initiates the atherosclerotic processes in early childhood.

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Keywords
Adiponectin • Atherosclerosis • Children • Intima media thickness • Obesity • Risk factors

1 Introduction

The formation of atherosclerotic plaque may begin, as confirmed by postmortem examination, in childhood and is associated with the existence, intensification, and interaction between numerous risk factors (Berenson et al. 1998). The obesity epidemic presents the most significant risk of atherogenesis in children and adolescents. The excessive amount of visceral adipose tissue plays a role in the development of hyperglycemia, insulin resistance, dyslipidemia, and arterial hypertension. Obesity is accompanied by a chronic inflammatory process. Cytokines secreted by visceral adipose tissue activate typical inflammatory cells (macrophages and lymphocytes), which induce a chronic inflammatory process and participate in the vascular endothelium lesion.

Atherosclerosis in children and adolescents is usually asymptomatic, thus, no unequivocal diagnostic criteria have been established so far. It may be helpful to assess the traditional cardiovascular disease risk factors in blood serum, such as the concentration of lipid profile components, glucose, and insulin. Recently, special attention has been given to the analysis of inflammatory biomarkers and endothelial dysfunction, both playing a significant role in the initial phase of atherogenesis. High sensitive C-reactive protein (hsCRP) is a well-established marker of an inflammatory process. Adiponectin is secreted by adipose tissue and is a protective factor for the endothelium. This function is attenuated in obese children. The measurement of carotid intima-media thickness is of practical importance. This parameter is a well-established marker of subclinical atherogenesis. The aim of the present study was to evaluate atherogenesis risk factors in obese children and adolescents.

2 Methods

We studied a group of 180 children and adolescents (aged 5–18 years) hospitalized in the Department of Pediatrics and Endocrinology of the Medical University of Warsaw in Poland: 122 children with simple obesity (52 girls, 70 boys, mean age 11.6 ± 3 years) and 58 healthy non-obese children (22 girls, 35 boys, mean age 11.7 ± 3 years). None of the subject had endocrine disorders, hereditary diseases, or systemic inflammatory diseases. All were non-smokers and did not receive any regular medication. The project received approval of the Ethics Committee of the Medical University of Warsaw, Poland.

Anthropometric measurements of all children were taken, including body height (cm), body weight (kg), waist and hip circumference (cm), thickness of 3 skinfolds (mm). The results of these measurements were used to determine body mass index (BMI), waist-to-hip ratio (WHR), waist-to-height ratio (WHtR), and the percentage of body percentage calculated using the Slaughter equations based on skinfold measurements (Slaughter et al. 1988).

Obesity was assessed using the International Obesity Task Force (IOTF) criteria (Cole et al. 2000). We used the LMS method, which normalizes the BMI skew distribution and expresses BMI as BMI SDS (body mass index standard deviation scores, also called BMI *z*-scores), which takes into account a relative weight adjusted for child age and sex to give meaningful comparisons (Cole 1990). The threshold of obesity was set at BMI SDS $\geq +2$. WHtR exceeding 0.5 was assumed to be a value indicating abdominal obesity. When determining the incidence of the metabolic syndrome, the 2007 International Diabetes Federation (IDF) criteria were used (Zimmet et al. 2007).

2.1 Biochemical Analysis

A concentration of adiponectin was measured with a radioimmunoassay (RIA) method, and that of acute-phase protein CRP with an enzymatic method in each subject after 12 h fasting. In obese children, the oral glucose tolerance test (OGTT) was performed to analyze glucose and insulin concentrations at 0, 30, 60, 90, and 120 min of the test (glucose were measured using the enzymatic method, whereas insulin concentrations were measured using the chemiluminescence and immunoenzymatic methods). We also measured lipid profiles including serum total cholesterol (TC), triglyceride (TG), and high density lipoprotein-cholesterol (HDL-C); LDL cholesterol was calculated using the Friedewald formula. These data were used to calculate the indicators of atherogenesis: total cholesterol/high-density lipoprotein cholesterol (TC/HDL-C), triglycerides/high-density lipoprotein cholesterol (TG/HDL-C), low-density lipoprotein cholesterol/high-density lipoprotein cholesterol (LDL-C/HDL-C), non-HDL (TC minus HDL-C), and the indicator of insulin resistance: HOMA (Homeostasis Model Assessment). The values of lipid metabolism were interpreted according to the American Heart Association recommendations (Hayman et al. 2007), while glucose levels were interpreted in accordance with the 2013 Polish Diabetes Association guidelines (PDA 2013). Hyperinsulinism was defined as fasting insulin level $\geq$ 15 μIU/ml, and/or maximum OGTT level $\geq$ 150 μIU/ml, and/or insulin level at 120 min of OGTT $\geq$ 75 μIU/ml. HOMA index to determine insulin resistance was defined to be $\geq$ 3 (Ten and Maclaren 2004). Normal values of TC/HDL-C were defined to be <5, TG/HDL-C < 3, LDL-C/HDL-C < 3, non-HDL (TC – HDL-C) $\geq$ 123 mg/dl (Millán et al. 2009; Olson et al. 2012; Srinivasan et al. 2006).

2.2 Blood Pressure

Blood pressure (BP) was measured in obese children. Every measurement was repeated three times at the right arm after a 10-min rest in the supine position and averaged. The cuff size was based on the length and circumference of the upper arm and was chosen individually. The results were evaluated using percentile charts for the population of Polish children. Hypertension was diagnosed when the values of systolic or diastolic blood pressure were above the 95th percentile; the mean blood pressure values between the 90th and 95th percentiles were defined as the border zone (Ostrowska-Nawarycz and Nawarycz 2007).

2.3 Carotid Artery Ultrasound

Carotid artery ultrasound examination was performed in 81 obese children and 32 patients from the control group. Measurements of intima-media thickness (IMT) were carried out using an ultrasound HDI 3000 scanner equipped with transducers of 12-MHz (ATL, Bothell, Washington DC). IMT measurements were performed thrice at the far wall of each common carotid artery about 1 cm from the bifurcation and the mean of these measurements was considered the final IMT value.

2.4 Statistical Analysis

The patients' data in each group and the data for each anthropometric and biochemical parameters were presented as means $\pm$ SD, minimal and maximal values. The investigated groups were compared with a *t*-test or analysis of variance ANOVA for data with normal distribution or Mann-Whitney U test for non-normal distribution. To determine the correlation between the two parameters Spearman's nonparametric correlation analysis was performed. A p-value < 0.05 was used to define statistically significant differences. The analysis of the results was prepared using Microsoft Excel 2010 spreadsheet. Detailed statistical calculations were performed using SPPS 19 software.

3 Results

The mean BMI of the obese children was 29.5 $\pm$ 4.9 kg/m^2, the mean SDS BMI was

Table 1 Biochemical parameters in obese and non-obese children

Variables	Obese (*n* = *122*)	Non-obese (*n* = *58*)
Fasting glucose (mg/dl)	82.5 ± 10.4	83.6 ± 10.3
Total cholesterol (mg/dl)	157.5 ± 22.4	176.9 ± 30.0**
HDL-cholesterol (mg/dl)	56.0 ± 11.9	44.4 ± 11.2**
LDL-cholesterol (mg/dl)	85.1 ± 24.2	105.8 ± 27.2**
Triglycerides (mg/dl)	76.9 ± 33.6	133.2 ± 62.9**
TC/HDL-C	2.9 ± 0.7	4.2 ± 1.2**
TG/HDL-C	1.5 ± 0.9	3.3 ± 2.1**
LDL-C/HDL-C	1.6 ± 0.7	2.5 ± 1.0**
Non-HDL	101.5 ± 25.5	132.5 ± 31.2**
CRP (mg/dl)	0.49 ± 0.20	0.45 ± 0.30
Adiponectin (μg/ml)	15.9 ± 6.6	13.1 ± 5.9*
IMT (mm)	0.55 ± 0.10	0.52 ± 0.10

LDL-cholesterol low-density lipoprotein cholesterol, *HDL-cholesterol* high-density lipoprotein cholesterol, *TC/HDL-C* total cholesterol/high-density lipoprotein cholesterol, *TG/HDL-C* triglycerides/high-density lipoprotein cholesterol, *LDL-C/HDL-C* low-density lipoprotein cholesterol/high-density lipoprotein cholesterol, *non-HDL* TC minus HDL-C, *CRP* C-reactive protein, *IMT* carotid intima-media thickness; *p < 0.01; **p < 0.001

2.8 ± 0.5. All children were found to have high body fat percentage, averaging 34.2 ± 5.0 %. The mean waist circumference was 90.3 ± 12.3 cm. In nearly all children their WHtR met the criteria for diagnosing abdominal obesity. The mean BMI of non-obese children was 18.7 ± 2.7 kg/m^2, the mean SDS BMI 0.0 ± 0.9.

For most biochemical parameters there were statistically significant differences between obese and non-obese patients (Table 1). The obese children had higher levels of TC, LDL-C, TG, and lower of HDL-C as compared with the children of proper weight. Obese children had also significantly elevated atherogenic indicators (TC/HDL-C, TG/HDL-C, LDL-C/HDL-C, and non-HDL). Twenty two percent of obese children had an increased value of TC (≥200 mg/dl), 16 % of them had elevated LDL-C (≥130 mg/dl), and 65 % had elevated TG (≥110 mg/dl). HDL-C below 40 mg/dl was found in 36 % of obese patients. As far as markers of atherogenesis are concerned, the indicator whose levels were most often elevated was non-HDL – in 61 % of the children. The other atherogenesis indicators: TC/HDL-C ≥ 5 was found in 21 % of obese children, TG/HDL-C in 16 %, and LDL-C/HDL-C in 11 %.

Impaired fasting glucose was found in 11 obese children: 7 girls and 4 boys. Abnormal glucose tolerance was diagnosed in 26 obese patients, including 11 girls and 15 boys. Glucose levels ≥ 200 mg% were found only in one obese boy (SDS BMI: 2.3).

Elevated fasting insulin levels ≥ 15 μIU/ml were found in 46.6 % of the children (21 girls and 34 boys). Abnormal insulin levels in the OGTT (≥150 μIU/ml) were observed in 19 patients at 30 min, 21 patients at 60 min, 18 patients at 90 min, and it was greater than ≥ 75 μIU/ml in 55 children at 120 min. The mean value of the insulin resistance indicator HOMA was 3.3 ± 2.0. HOMA values ≥ 3 showing severe insulin resistance were found in 46 % of obese children.

In the group of obese children, 6.1 % had systolic blood pressure slightly above the 95th percentile, and 14.2 % had diastolic blood pressure slightly above the 95th percentile. The mean adiponectin concentration in obese children was significantly lower than in the control group (13.1 μg/ml *vs*. 15.9 μg/ml; p = 0.004). Slightly higher values were found in obese girls as compared with obese boys (13.4 μg/ml *vs*. 12.9 μg/ml; p = 0.775). Correlations between adiponectin level and anthropometric and biochemical parameters are presented in Table 2. In children with proper body weight and obese, evaluated together, adiponectin concentration negatively correlated with BMI, BMI SDS (p < 0.05). Moreover, adiponectin concentration positively correlated with HDL-C and negatively with all the indicators of atherogenesis: TC/HDL-

Table 2 Correlations between the level of adiponectin, on the one side and anthropometric parameters, lipid metabolism parameters, and atherogenic indices in all children and non-obese children, on the other side

Correlating variables	All children	Non obese children
Age (year)	0.002	−0.006
Height (cm)	−0.059	−0.095
Body weight (kg)	−0.135§	−0.063
Body mass index (kg/m^2)	−0.182*	−0.059
BMI SDS	−0.159*	0.003
Waist circumference (cm)	−0.148§	−0.040
Hip circumference (cm)	−0.120	−0.057
Waist-to-hip ratio (WHR)	−0.101	0.054
Waist-to-height ratio (WHtR)	−0.123	0.013
%Body fat (skinfold)	−0.134§	−0.033
Total cholesterol (mg/dl)	−0.084	0.041
HDL-cholesterol (mg/dl)	0.220**	0.183*
LDL-cholesterol (mg/dl)	−0.115	0.008
Triglycerides (mg/dl)	−0.147§	−0.048
TC/HDL-C	−0.206**	−0.099
TG/HDL-C	−0.190*	−0.109
LDL-C/HDL-C	−0.192*	−0.095
Non-HDL	−0.156*	−0.024
CRP (mg/dl)	−0.195*	−0.111

BMI SDS body mass index standard deviation scores, also called BMI *z*-scores, *LDL-cholesterol* low-density lipoprotein cholesterol, *HDL-cholesterol* high-density lipoprotein cholesterol, *TG/HDL-C* triglycerides/high-density lipoprotein cholesterol, *LDL-C/HDL-C* low-density lipoprotein cholesterol/high-density lipoprotein cholesterol, *TC/HDL-C* total cholesterol/high-density lipoprotein cholesterol, *TG/HDL-C* triglycerides/high-density lipoprotein cholesterol, *non-HDL* TC minus HDL-C, *CRP* C-reactive protein; *$p < 0.05$; ** $p < 0.01$; §$0.05 < p < 0.09$ – borderline significance

C, TG/HDL-C, LDL-C/HDL-C, and non-HDL, and with CRP. In the group of obese children alone, a weak positive correlation of adiponectin concentration with HDL-C concentration was found. A negative correlation was found between adiponectin and insulin at 90 min of the OGTT test ($r = -0.18$, $p = 0.05$). No significant correlations were identified between adiponectin and fasting or OGTT glucose levels, HOMA, BP, and carotid IMT. Furthermore, a multiple regression analysis using adiponectin as an dependent variable and age, sex, degree of obesity (BMI, SDS BMI, and percent of the body fat), waist circumference, HDL-C, TG, fasting insulin, and HOMA as independent variables resulted only in a trend toward negative correlation with fasting insulin ($p = 0.07$).

The mean carotid IMT in the obese group was 0.55 ± 0.1 mm, and in the group of children with normal body weight it was 0.52 ± 0.1 mm; the difference between the two groups was insignificant ($p = 0.268$). Carotid IMT values in the obese group correlated with glucose level at 90 min ($r = 0.26$; $p = 0.022$) and at 120 min ($r = 0.23$; $p = 0.044$) of the OGTT test and with systolic ($r = 0.30$; $p = 0.014$) (Fig. 1) and diastolic blood pressure ($r = 0.24$; $p = 0.049$).

The metabolic syndrome was diagnosed in 17 out of the 84 obese patients > 10 years old (20.2 % of the group). Those children were found to have lower mean adiponectin concentrations (11.3 *vs.* 13.13 μg/ml; $p = 0.314$) and higher mean IMT values (0.61 *vs.* 0.55 mm; $p = 0.031$) as compared with those obese children who did not meet the criteria of the metabolic syndrome (Fig. 2).

4 Discussion

Cardiovascular disease constitutes to be one of the main causes of morbidity and mortality in the

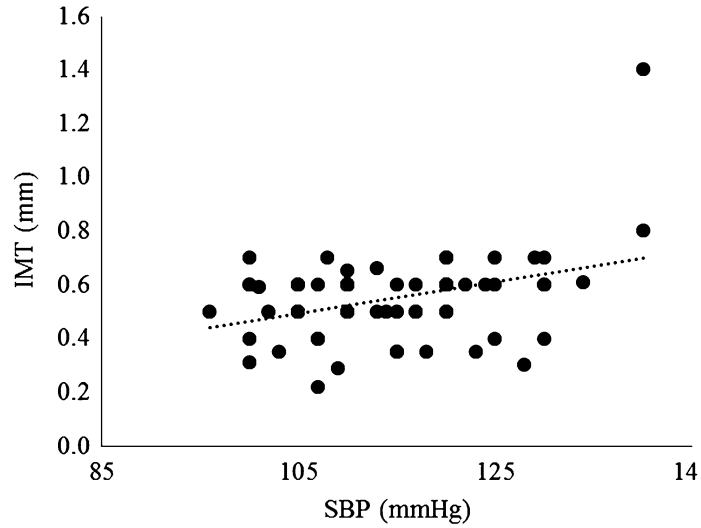

Fig. 1 Correlation between carotid intima-media thickness (IMT) and systolic blood pressure (SBP) in obese children

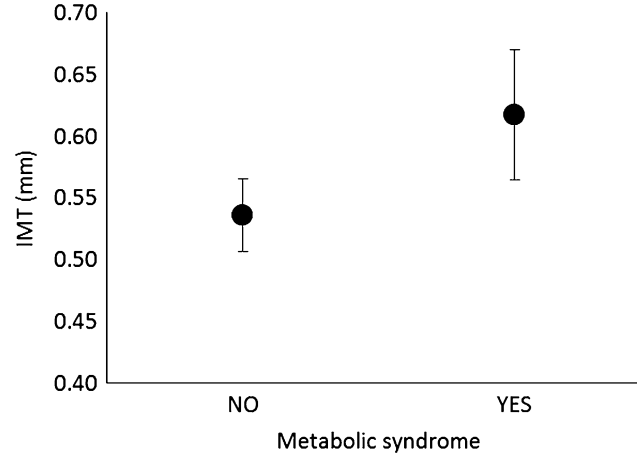

Fig. 2 Carotid intima-media thickness (IMT) in obese children older than 10 years of age, with and without metabolic syndrome. Data are means and 95 % confidence intervals

world. It is triggered by the formation of atherosclerotic plaque, whose development is dependent on the existence, intensification, and interaction between several risk factors. The obesity epidemic presents a major threat for public health. Accompanied by lipid and carbohydrate abnormalities, arterial hypertension and a chronic inflammatory process, may damage arterial walls in early life contributing to the development of cardiovascular disease in adulthood.

Lipid abnormalities have been traditionally considered as risk factors in atherogenesis for many years. In comparison to their lean peers, obese children have a higher concentration of triglyceride, total- and LDL-cholesterol, while their HDL-cholesterol concentration is lower (Reinehr and Andler 2004). Hypertriglyceridemia favors the formation of small dense LDL particles. These particles demonstrate a prolonged half-life and infiltrate the walls of blood vessels more intensely. They are not only more susceptible to oxidation, but also they are taken up and assimilated by macrophages more effectively. The constellation of an increased triglyceride concentration, a higher number of plasma LDL-cholesterol particles, and a lower

HDL-cholesterol concentration constitute the phenotype of atherogenic dyslipidemia, commonly seen in obese children. The measurement of non-HDL cholesterol is a simpler and better screening tool of cardiovascular risk. This parameter reflects the concentration of all atherogenic lipoproteins including low-density lipoproteins (LDL), very-low density lipoprotein (VLDL) and their remnants, intermediate-density lipoprotein (IDL-C), and lipoprotein A. The level of non-HDL cholesterol correlates closely with the concentration of apolipoprotein B. It is a well-established marker of subclinical atherogenesis, significantly better than LDL-cholesterol. Long-term studies of children and adolescents have shown that assessing the risk of endothelial dysfunction in adulthood can be determined by means of carotid intima-media thickness (Srinivasan et al. 2006). The present study confirms an abnormal lipid profile in obese patients, as a higher level of non-HDL was observed in 61 % of children.

Low grade, subclinical inflammation is of primary importance in atherogenesis (El-Shorbagy and Ghoname 2010). Mediators of the inflammatory reaction, released from adipocytes and stromal macrophages, directly affect the vascular endothelium leading to its dysfunction being the first stage of atherogenesis. C-reactive protein (CRP) is a good marker of an inflammatory process; its synthesis in the liver is stimulated by the interleukins IL-1 and IL-6 and by tumor necrosis factor-α. CRP directly affects endothelial cells, macrophages, monocytes, and smooth muscles cells. It also activates the complement system and induces prothrombotic activity. Owing to its high sensitivity, the CRP assay could be used in the assessment of a subclinical inflammatory process, associated with obesity and atherogenesis not only in adults but also in children. CRP concentration is higher in obese and overweight children and rises if it coexists with type 2 diabetes, the metabolic syndrome and non-alcoholic fatty liver disease. It correlates positively with body weight, BMI, waist circumference, insulin, HOMA, triglycerides, and arterial blood pressure, and correlates negatively with HDL-cholesterol (El-Shorbagy and Ghoname 2010; Kitosis et al. 2013). In the present study, concentration of CRP was measured by means of a conventional enzymatic assay, which explains the absence of statistically relevant differences in its average concentration between the obese and non–obese groups. Despite the low-sensitivity of the assay applied, the concentration of CRP correlated negatively with the adiponectin level in children with proper body weight and obese children pooled together, which points to CRP participation in the development of an inflammatory process. This observation is consistent with other published data. The clinical studies have shown that the level of adiponectin correlates with the markers of endothelial dysfunction and of an inflammatory process: negatively with TNFα, E-selectin, IL-1, monocyte chemotactic protein-1 (MCP-1), and correlates positively with the bioactivity of nitric oxide (NO) (El-Mesallamy et al. 2011; Belo et al. 2013).

Adiponectin exerts an anti-inflammatory effect on the vascular wall and reduces atherosclerosis. Its influence on endothelial cells is complex. It accumulates in the damaged vessel, suppresses adhesion molecule expression, reduces macrophages transformation in foam cells, inhibits proliferation and migration of vascular smooth muscles cells, and enhances NO production. Anti-inflammatory effects of adiponectin are associated with blocking of the nuclear factor κβ signaling by inhibiting the expression of Il-6 and TNFα (Ouchi et al. 2004; Chen et al. 2003). Apart from its influence on the structure and function of vascular endothelium cells, adiponectin exhibits antiatherosclerotic properties by modulating cardiovascular risk factors, e.g., it regulates the lipid profile, which was also demonstrated in the present study. In obese children with low adiponectin concentration, vascular walls are less protected. Numerous studies have shown that hypoadiponectinemia is an independent cardiovascular risk factor (Frystyk et al. 2007).

A useful, noninvasive method to assess early atherosclerosis is the measurement of carotid IMT with ultrasound imaging. IMT results correspond with the histological changes and reflect

the severity and extent of atherosclerosis in other arteries. High IMT values were found in adults with obesity, diabetes, hypertension, and a smoking habit. Increased carotid IMT values are related to the well-established cardiovascular risk factors and precede the occurrence of myocardial infarction and stroke (Reinehr et al. 2006). Children with familial hipercholesterolemia, type 1 diabetes, hypertension, and chronic kidney disease have significantly increased IMT. A majority of reports show that IMT also is increased in children with obesity. In meta-analysis of the studies published in the years 2000–2001, Silva et al. (2012) failed to identify any crucial differences in IMT between the obese and non-obese children only in 4 out of the 16 studies. In addition, Tounian et al. (2001) have pointed out that, although no significant differences in IMT were revealed, obese children exhibited increased stiffness of elastic arteries, lower brachial artery distensibility, and lower flow-mediated and glyceryl trinitrate-mediated dilation. The above-mentioned studies indicate that although no significant difference in IMT could be observed, obese children have some endothelial dysfunction and the differences in IMT might be revealed at later age. The Bogalusa Heart Study and the Muscatine Study have shown that exposure to biochemical and hemodynamic obesity-related disorders in childhood are directly associated with increased IMT values in adulthood (Berenson and Bogalusa Heart Study Investigators 2001; Davis et al. 2010). These findings might be relevant to the absence of significant differences in IMT between the obese and non-obese groups in the present study. The patient age, duration and level of obesity, and other cardiovascular risk factors might also influence the IMT values. As shown by Reinehr et al. (2006), IMT values correlate with BMI, SDS BMI, fasting glucose concentration, hsCRP, and arterial hypertension, but not with age, insulin level, and lipid profile. The study of Giannini et al. (2008) has shown that IMT values are not statistically different in BMI percentile and waist circumference percentile groups, but depend on insulin resistance. The thickness of IMT correlates with fasting the insulin concentration, the homeostatic model assessment (HOMA), the fasting glucose to insulin ratio (FGIR), the quantitative insulin sensitivity check index (QUICKI), and the Matsuda index of insulin sensitivity, while the association of IMT with arterial hypertension, LDL-C, and hsCRP is statistically irrelevant. The present study demonstrates that IMT values depended on glucose concentration and in the first place on systolic blood pressure, which is a key cardiovascular disease factor. The IMT is affected by the intensity of cardiovascular risk factors (Stauboli et al. 2012). The study of children aged 10 years and older fulfilling the criteria of the International Diabetes Federation (IDF) Worldwide Definition of the Metabolic Syndrome has revealed significantly increased IMT as compared with obese children not fulfilling the metabolic syndrome criteria (Zimmet et al. 2007).

5 Conclusions

Abdominal obesity and the accompanying metabolic and hemodynamic components of metabolic syndrome contribute to the early development of atherosclerosis, assessed by the measurement of carotid intima-media thickness (IMT). High systolic blood pressure plays a major role in changing the carotid intima-media complex thickness. Adiponectin may participate in atherogenesis through its impact on lipid profile and inflammation. The evaluation of the presence of traditional and emergent cardiovascular factors might prove helpful in identifying a group of children who are at risk for developing atherosclerosis.

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

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Nutrition and Immune System in Children with Simple Obesity

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Abstract

The purpose of this study was to evaluate dietary factors in nutrition influencing the immune system of children and teenagers suffering from simple obesity. The study involved 100 children and teenagers aged 7–18 with simple obesity. Nutritional data were obtained from 3-day food records. The consumed nutrients, including immunomodulators and immunostimulants, were estimated based on the nutrition interview. The results were compared with the nutritional norms. On average, the proportion of n-6:n-3 fatty acids equalled 10:1. Among the amino acids, the highest intake values in the diet were observed for glutamine (13,694.6 mg/day). The study demonstrates inadequate intake levels of iron (73 % of recommended dietary allowance, RDA), vitamin C (65 % of RDA), and vitamin D (11 % of RDA) taking into account the median values for the entire study group. The median daily intake of other nutrients exceeded the RDA values. The diets of the participants in this study were not properly balanced with respect to immunomodulators, which may contribute to the occurrence of immunological disorders and immunodeficiency in this group of patients.

Keywords

Children • Diet • Obesity • Immunomodulators • Immunostimulants • Immunological disorders

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1 Introduction

Nutritional status has a significant effect on the functioning of the body's immune system. Previous studies have shown that infections are more common in obese subjects than in those with body mass within normal ranges. Obesity visibly manifests itself through changes in immunity, particularly in cellular responses (Matarese and La Cava 2005). Moreover, the capability of T and B lymphocytes to respond to pathogens is lower in obese persons. Human studies have demonstrated that excessive body mass is correlated with impaired activity of immunocomponent cells (Samartin and Chandra 2001).

Both excess and deficiency of various microcomponents in the diet may influence immunity. Moderation and appropriate intake of most nutrients are required to ensure optimal immunity (Rautava and Walker 2009). Deficiencies of nutrients such as iron or zinc in obese persons, which lead to changes in the immune system, can be reverted with supplementation and balancing the concentration of these components in the organism (Samartin and Chandra 2001).

In recent years, studies have confirmed the hypothesis that obesity is a significant factor contributing to the development of asthma. Many cross-sectional studies have been conducted including children, teenagers and adults, on the impact of obesity on the occurrence of asthma. In acute cases of asthma, excessive body mass seems to be a significant factor contributing to the development of the disease. High body mass index (BMI) correlates with airway hyperreactivity, which, in turn, contributes to the exacerbation of the disease. After reduction of body mass, an improvement in the asthma control is observed (Matarese and La Cava 2005; Shore and Johnston 2006).

The definition of "immunonutrition", i.e., immunomodulatory diet, requires that diet modulates the immune system by influencing its functions through various nutritional components. Dietary factors in nutrition influencing the immune system notably include glutamine, arginine, omega-3 (n-3) and omega-6 (n-6) fatty acids, zinc, iron, selenium, vitamins: E, A, C, D, lycopene, and the components of breast milk (Wichers 2009; McCowen and Bistrian 2003). The purpose of the present study was to evaluate immunomodulators and immunostimulants in the diet of children and teenagers suffering from simple obesity.

2 Methods

The study was performed in conformity with the Declaration of Helsinki for Human Experimentation and was approved by a local Ethics Committee. The investigation involved a group of children and teenagers, aged 7–18 ($n = 100$), diagnosed with simple obesity. BMI was calculated as weight (kg)/height (m^2), SDS BMI adjusted for age and sex calculated using normative data. Obesity was defined as SDS BMI of more than +2. The BMI of all obese subjects was $>$ 95th percentile for age and sex reference values.

Nutritional data were obtained from 3-day food records. The food records included at least one weekend day. The history regarding the way of feeding during the first 12 months of life and chronic diseases, including allergies and asthma, and supplementation of the diet were provided by the parents or legal guardians.

The energetic and nutritional value of the consumed foods was estimated, including, in particular, the percentage of intaken amino acids (arginine and glutamine), minerals (zinc and iron), vitamins (C, E, A, and D), and omega-3 (n-3) and omega-6 (n-6) fatty acids. The intake of selenium was calculated based on the American tables of composition and nutritional values of foods (USDA 2011). The intake of lycopene was calculated based on the study by Hamulka and Wawrzyniak (2004). The data obtained from each patient were compared with the recommended dietary allowances (RDA) and adequate intake (AI) for vitamin E for both genders and for different age groups (Jarosz and Bulchak–Jachymczyk 2012), which served as a basis for calculation of the rate of conformity.

Data were presented as medians and the 25–75th percentile interquartile ranges (IQR). The level of statistical significance was established at $p < 0.05$. Statistical analysis was conducted using a commercial Statistica 9.0 software package.

3 Results

The mean rate of conformity with the current recommendation concerning the energy intake for the entire group was 73 %. The diet of children aged 7–9 was in most cases in accordance with the recommendation. The lowest rates of conformity were observed in teenagers aged 16–18 (Table 1).

The median intake of omega-3 polyunsaturated fatty acids was 0.8 g/day and of omega-6 was 8.2 g/day. The mean proportion of n-6:n-3 fatty acids equalled 10:1. The analysis of diets showed that the median intake of arginine in the study group was 3,596.5 mg/day. The median values of glutamine consumed by the children was 13,694.6 mg/day. The median intake of lycopene in the study group was 1.2 mg/day, which accounted for a 20 % rate of conformity with the recommended values (Table 2).

The median intake of zinc in the studied group was 8.4 mg/day (99 % of RDA). The rate of conformity with recommended values with regard to selenium was 240 % (median intake 107.8 μg/day). Nutrient deficiencies were mostly observed in regard to iron, which was provided with diet at a level of 73 % of RDA. The mean intake of fat-soluble vitamins was 104 % of RDA for vitamin A and 77 % of AI (Adequate Intake) for vitamin E. The rate of conformity with the recommended allowance for vitamin C was 65 % for the entire study group. All patients reported inadequate intake of vitamin D from diet (11 % of RDA) (Table 3). None of the patients used vitamins or minerals supplements.

A significant correlation was observed between body mass and consumption of omega-6 polyunsaturated fatty acids. Children and teenagers whose body mass was higher consumed significantly more omega-6 fatty acids ($r = 0.322$, $p < 0.05$). The rate of conformity with the recommended values of zinc correlated negatively with body mass (Fig. 1). Similar correlations were observed in the case of conformity with the recommended values of energy intake and components such as fat, iron, and selenium (Table 4).

Table 1 Energy and macronutrients of consumed food in diets in different age and sex groups, compared with the recommended values

Gender/age	Energy (kcal) (% *RDA*) [*IQR*]	Protein (g) (%*RDA*) [*IQR*]	Fat (g) (%*RDA*) [*IQR*]	Carbohydrates (g) (%*RDA*) [*IQR*]
Girls & Boys 7–9 years	1,642.0 (91 %)	62.0 (206 %)	60.2 (100 %)	218.4 (168 %)
(*n = 22*)	[1,396.0–1811.0]	[49.4–68.9]	[56.4–78.5]	[175.9–251.4]
Girls 10–12 years	1,704.0 (81 %)	62.9 (153 %)	69.5 (99 %)	213.0 (164 %)
(*n = 20*)	[1,343.0–2076.0]	[55.0–76.8]	[45.9–83.1]	[189.5–274.8]
Boys 10–12 years	1,547.0 (64 %)	60.1 (143 %)	65.3 (82 %)	197.3 (152 %)
(*n = 21*)	[1,445.0–1814.0]	[49.0–70.9]	[53.1–80.9]	[131.0–243.3]
Girls 13–15 years	1,942.0 (79 %)	65.6 (117 %)	93.9 (114 %)	245.2 (189 %)
(*n = 7*)	[1,808.0–2,347.0]	[61.5–72.5]	[62.9–113.1]	[205.5–309.9]
Boys 13–15 years	1,619.0 (54 %)	69.8 (120 %)	51.3 (51 %)	198.8 (153 %)
(*n = 12*)	[1,298.0–1743.0]	[55.9–86.8]	[38.8–78.1]	[169.2–275.5]
Girls 16–18 years	1,783.0 (71 %)	64.3 (121 %)	63.1 (76 %)	228.1 (175 %)
(*n = 10*)	[1,372.0–1,906.0]	[55.5–84.3]	[52.5–82.9]	[171.7–248.9]
Boys 16–18 years	1,864.0 (55 %)	67.0 (104 %)	59.3 (52 %)	256.6 (197 %)
(*n = 8*)	[1,441.0–2,106.0]	[87.0–111.0]	[47.6–79.8]	[218.6–304.1]

Data are medians and interquartile ranges (IQR, 25th–75th percentile); *RDA* recommended dietary allowances

Table 2 Immunostimulants in diets in different gender/age groups

Gender/age	Omega-3 (g) [*IQR*]	Omega-6 (g) [*IQR*]	Glutamine (mg) [*IQR*]	Arginine (mg) [*IQR*]	Lycopene (mg) [*IQR*]
Girls & Boys 7–9 years	0.7	6.1	13,481.6	3,596.5	1.5
(*n* = *22*)	[0.5–1.2]	[4.8–8.8]	[10,814.0–14,911.0]	[3,084.0– 4,187.0]	[0.0–3.4]
Girls 10–12 years	0.7	9.8	14,187.6	3,517.1	1.5
(*n* = *20*)	[0.5–1.1]	[5.6–12.9]	[11,902.7–16,993.5]	[3,175.5–4,277.1]	[0.3–4.8]
Boys 10–12 years	0.8	8.4	11,811.6	3,630.1	0.7
(*n* = *21*)	[0.7–1.5]	[5.4–13.2]	[10,451.4–14,899.3]	[3,036.4–4,162.1]	[0.0–1.8]
Girls 13–15 years	0.9	9.2	14,989.3	3,838.5	0.0
(*n* = *7*)	[0.7–1.0]	[8.0–19.2]	[11,687.8–16,531.1]	[3,517.9–4,161.1]	[0.0–1.4]
Boys 13–15 years	0.8	6.8	15,103.1	3,498.6	2.6
(*n* = *12*)	[0.7–1.2]	[4.5–10.2]	[12,776.1–19,283.9]	[2,999.8–4,822.4]	[0.0–5.5]
Girls 16–18 years	1.1	11.0	13,122.8	3,324.3	1.5
(*n* = *10*)	[0.8–1.4]	[8.1–13.0]	[11,228.5–17,422.3]	[2,291.1–4,459.4]	[0.3–1.8]
Boys 16–18 years	1.1	11.8	14,540.4	3,960.2	0.9
(*n* = *8*)	[0.8–1.6]	[5.2–17.8]	[11,526.9–15,497.9]	[3,310.8–4,665.5]	[0.0–2.5]

Data are medians and interquartile ranges (IQR, 25th–75th percentile)

Table 3 Vitamins and minerals of immunostimulatory function in diets in different gender and age groups, compared with the recommended values

Gender/age	Vitamin A (pg) (% RDA) [*IQR*]	Vitamin E (mg) (%AI) [*IQR*]	Vitamin C (mg) (% RDA) [*IQR*]	Vitamin D (μg) (%RDA) [*IQR*]	Zinc (mg) (% RDA) [*IQR*]	Iron (mg) (% RDA) [*IQR*]	Selenium (μg) (%RDA) [*IQR*]
Girls & Boys 7–9 years	704.0 (141 %)	6.0 (86 %)	27.3 (55 %)	1.5 (10 %)	8.4 (168 %)	8.8 (88 %)	106.3 (354 %)
(*n* = *22*)	[263.7–1,172.7]	[4.6–7.6]	[20.8–41.2]	[1.0–1.8]	[6.4–10.2]	[6.6–11.0]	[81.8–114.5]
Girls 10–12 years	786.0 (131 %)	6.7 (84 %)	43.6 (87 %)	1.7 (11 %)	7.9 (99 %)	8.2 (82 %)	104.7 (262 %)
(*n* = *20*)	[489.7–1,497.7]	[4.9–9.5]	[24.9–53.7]	[1.2–3.0]	[6.5–9.6]	[6.1–10.5]	[83.8–127.6]
Boys 10–12 years	667.0 (111 %)	6.4 (64 %)	33.4 (67 %)	1.6 (11 %)	7.2 (90 %)	7.9 (79 %)	96.5 (241 %)
(*n* = *21*)	[270.0–1,107.0]	[4.0–8.7]	[20.5–52.4]	[1.1–2.1]	[6.4–8.4]	[6.0–9.3]	[79.0–122.0]
Girls 13–15 years	409.0 (58 %)	9.4 (117 %)	43.3 (67 %)	1.5 (10 %)	8.7 (96 %)	7.9 (53 %)	97.8 (178 %)
(*n* = *7*)	[378.0–1,026.0]	[6.8–15.4]	[35.7–132.4]	[1.3–2.0]	[8.2–9.7]	[7.5–10.0]	[91.9–98.3]
Boys 13–15 years	674.5 (75 %)	5.5 (55 %)	36.0 (48 %)	1.6 (11 %)	8.9 (81 %)	8.7 (72 %)	115.6 (210 %)
(*n* = *12*)	[434.0–1,039.2]	[3.4–8.5]	[16.7–40.8]	[0.9–2.1]	[7.9–11.3]	[7.8–9.9]	[101.2–152.5]
Girls 16–18 years	735.5 (105 %)	5.8 (73 %)	44.0 (68 %)	2.0 (13 %)	8.6 (95 %)	8.1 (54 %)	119.2 (217 %)
(*n* = *10*)	[469.5–1,186.7]	[3.1–8.2]	[10.2–51.8]	[1.1–2.4]	[6.0–11.1]	[7.8–9.8]	[83.5–143.3]
Boys 16–18 years	658.5 (73 %)	6.8 (68 %)	70.1 (93 %)	1.4 (9 %)	9.7 (88 %)	8.7 (72 %)	118.0 (215 %)
(*n* = *8*)	[562.7–932.5]	[5.0–9.6]	[51.8–88.1]	[1.0–1.7]	[8.5–10.1]	[7.0–9.5]	[114.1–127.5]

Data are medians and interquartile ranges (IQR, 25–75th percentile); *RDA* recommended dietary allowances, *AI* adequati intake

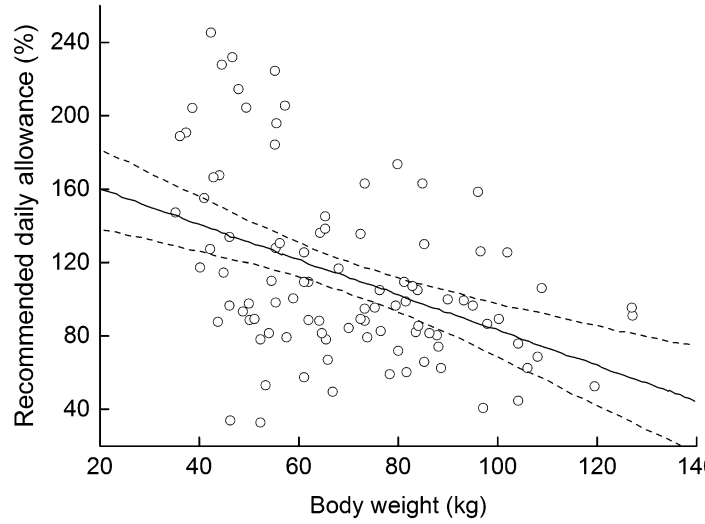

Fig. 1 The correlation between body mass and rate of conformity with recommended values for zinc ($r = -0.433$; $p < 0.05$)

Table 4 Relationship between body mass and selected nutrients

Component	r	p
Body mass *vs*. energy	−0.361	0.001
Body mass *vs*. fat	−0.288	0.004
Body mass *vs*. iron	−0.264	0.008
Body mass *vs*. selenium	−0.339	0.001

Among all the participants, 88 % of the children were breastfed until they were 12 months old. After that time, 48 % of mothers continued to breastfeed for some time, 10 % continued to breastfeed for more than 2 years in total, and 5 % for more than 3 years. About 40 % of children, whose mothers declared that they were breastfeeding, were diagnosed with food or airborne allergies. A small group of participants (3 %) was diagnosed with asthma.

4 Discussion

Over recent years, the number of subjects diagnosed as overweight or obese has been gradually increasing. Excess of body fat leads to various health consequences, which are often life-threatening. Child and teenage obesity may result in cardiovascular disease and carbohydrate and lipid metabolism disorders in adult life. Additionally, fat secretes pro-inflammatory substances which impair the immune system function.

The analysis of diets of the enrolled subjects demonstrated that the n-6:n-3 fatty acids ratio in daily diet was incorrect in most of the subjects examined. Excessive intake of omega-6 fatty acids, coupled with a high ratio of n-6:n-3, plays a role in the pathogenesis of many diseases, including cardiovascular diseases, tumors, inflammatory diseases, and autoimmune disorders. Intake of high amounts of omega-3 fatty acids, coupled with a lower ratio of n-6:n-3, inhibits the progression of cardiovascular diseases. Lowering this ratio to 4:1, as a part of secondary prevention of vascular disease, leads to a decrease in mortality by 70 %. The recommended ratio of 5:1 has a positive influence on the treatment of asthma, whereas the ratio of 10:1 does not show any further positive implications (Simopoulos 2002). Studies have shown that the mean ratio of polyunsaturated fatty acids in the diets of children and teenagers suffering from obesity is 10:1, which means that the potentially pro-inflammatory n-6 acids constituted the majority in these diets. Dry and Vincent (1991) have conducted an analysis of the influence of omega-3 acids on the progression of asthma. Intake of n-3 acids has been associated with a relatively less frequent occurrence of inflammatory diseases. The first effects of the

administered treatment were observed after only 9 months. The influence of n-3 acids on the progression of asthma was particularly important as allergies and asthma often coexist with obesity. The present study demonstrates that 3 % of enrolled children suffered from asthma, and just under 40 % suffered from various allergies that may increase the risk of developing asthma later in life.

The immunomodulatory properties of arginine result primarily from its role in the formation of nitrogen oxide and synthesis of glutathione (Rautava and Walker 2009). Glutamine, on the other hand, plays an important role in T lymphocyte proliferation and differentiation of B lymphocytes (Newsholme et al. 1999; Roth 2007). The present study demonstrates that median intake of arginine in the study group was of 3,596.5 mg/day and glutamine – 13,694.6 mg/day. The high intake of proteins may suggest that the consumption of these amino acids was also high. However, the lack of recommendations for the daily consumption makes it impossible to conduct a detailed analysis of the data.

Insufficient intake of iron may have life-threatening consequences. The most common presentation of this deficiency, in addition to various disorders of the immune systems, is iron deficiency anaemia (Weiss 2005). The results of the present study demonstrate insufficient intake of iron in the diet of children and teenagers. The conformity of the median for the entire study group with the daily recommended values was 73 %. Experiments conducted in animal models have demonstrated that insufficient iron intake results in the impairment of functioning of natural killer cells and of B lymphocytes responsible for the production of antibodies (Kuvibidila and Baliga 2002). A particularly important aspect, especially during the child developmental period, is iron deficiency that may impair the metabolism of neurotransmitters and the transport of oxygen, which may lead to improper functioning of the central nervous system and impairment of cognitive functions in children (Walter 2003).

Among the presented results, interestingly, the recommended intake values for selenium were significantly exceeded. The median value for the entire group of participants amounted to 240 % of RDA. Such high values may result from the fact that the estimation of this element in the diet was based on the databases of the American tables of composition and nutritive values of food products (USDA 2011). Nevertheless, excess intake of selenium may be toxic to the human organism. Studies conducted in rats by LeBoeuf and Hoekstra (1983) have demonstrated that an excess intake of selenium results in adaptive changes in the liver due to increased oxidation of glutathione. Cells of the immune system are particularly sensitive to deficiency of this glutathione. Its antioxidant properties protect immunological components against the destructive properties of free radicals.

Insufficient intake of antioxidant vitamins may have negative implications for the functioning of the immune system. The present study demonstrates insufficient intake of vitamin C (65 % of RDA for the entire study group). Deficiency of ascorbic acid may impair the process of T lymphocyte proliferation and cytotoxicity of neutrophils and Tc lymphocytes; the effects revert to normal with the correction of insufficient intake of vitamin C (Noh et al. 2005). We also found a lower than recommended intake of vitamin E by the participants of the study. The conformity of median for this component for the entire study group was 77 % of AI. Vitamin E, as an antioxidant, protects against oxidative stress. Insufficient intake of this component impairs the defense against potential pathogens (Mitchell et al. 2003). Likewise, the present study demonstrates an insufficient intake of vitamin D by children and adolescent (11 % of RDA). In addition to its role in maintaining calcium balance, vitamin D has a number of immune regulatory properties. The active form of vitamin D (1,25-dihydroxyvitamin D3) modulates Th1 and Th2 cell responses. Vitamin D deficiency is associated with increased susceptibility to respiratory tract infection in children and it also appears to be linked to the development of immune-mediated diseases (Rautava and Walker 2009).

The values of lycopene intake differ in various geographical regions. The estimated mean

intake of this component in the USA falls within the range of 3.7–16.2 mg/day. In Canada, this value fluctuates around 25.2 mg/day, and in Germany – around 1.3 mg/day (Rao and Rao 2007). The present study demonstrates that the median intake of lycopene in children and teenagers suffering from obesity was 1.2 mg/day (20 % of RDA). As a result of the photoprotective properties of lycopene, its insufficient intake may increase the destructive properties of UV light, which leads to weakening of the immune system. Additionally, insufficient lycopene intake may result in a simultaneous decrease in the number of natural killer cells and in a decrease in their cytotoxic capabilities (Chew 1993).

The majority of participants in the present study were breastfed during infancy. The percentage if children who were not breastfed was 12 %. Choosing non-natural feeding is primarily associated with various disorders of immunological functions of the child's organism. Additionally, it increases the risk of child obesity and type 1 and type 2 diabetes (Stuebe 2009). Moreover, breastfeeding at the early stages of life decreases the risk of obesity in adults (Owen et al. 2005). Due to the immunomodulatory properties of breast milk, it supports the development of immunological mechanisms in infants. Breast milk constitutes an optimal food for a newborn, as it has antibacterial properties and contains components that facilitate development of the immune system (Brandtzaeg 2002).

Excessive weight and obesity are tightly associated with the functioning of the immune system. Excess body fat is significantly correlated with the changes in immunological response. It weakens immunity against bacterial and viral pathogens (Matarese and La Cava 2005). Additionally, fat produces pro-inflammatory cytokines contributing to the occurrence of chronic inflammations (Samartin and Chandra 2001). Correct functioning of the immune system plays a crucial role in the process of the reduction of weight. Reduction of body mass and intake of all the necessary nutrients, including immunomodulators and immunostimulants, may significantly contribute to the improvement of the health condition.

To summarize, in the present study we demonstrate that the diet of children and teenagers suffering from simple obesity was not properly balanced in regard to nutrients and immunomodulators. Insufficient intake of crucial immunomodulatory and immunostimulating nutrients (such as iron, vitamin E, vitamin C, and vitamin D) and an incorrect ratio of polyunsaturated n-6: n-3 fatty acids, may impair the function of the immune system of children and teenagers later in life.

Conflict of Interest The authors declare that they have no conflict of interest.

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The Influence of Shockwave Therapy on Orthodontic Tooth Movement Induced in the Rat

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Abstract

Shockwave therapy is used in medicine due to its ability to stimulate healing processes. The application of orthodontic force evokes an inflammatory reaction resulting in tooth movement. Shockwave therapy might have an effect on both inflammatory and periodonal ligament cytokine profiles. Our aim was to evaluate the fluctuations of different inflammatory cytokines after orthodontic force induction with and without shockwave therapy. An orthodontic appliance was applied between the rats' molars and incisors. In conjunction with the commencement of orthodontic force, the rats were treated with a single episode of 1000 shock waves and the gingival crevicular fluid was collected for 3 days. The expression and concentration of different cytokines was evaluated by a commercial 4-multiplex fluorescent bead-based immunoassay. The level of all cytokines displayed a similar trend in both shockwave-treated and untreated groups; the concentration peaked on the first day and declined thereafter. In all cases, however, the cytokine levels were smaller in the shockwave-treated than in untreated animals; a significant difference was found for sRANKL and borderline difference for IL-6 on Day 1. We conclude that shockwave therapy during the induction of orthodontic tooth movement influences the expression of inflammatory cytokines.

Keywords

Cytokines • Gingival crevicular fluid • Healing • Inflammation • Orthodontic tooth movement • Shockwave therapy • Teeth

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1 Introduction

Shock waves have been applied clinically for the first time in renal stone therapy (Graff et al. 1988), where they were casually noted to have an effect on the iliac bone with primary osteocyte damage, followed by osteoblastic stimulation. Consequently, shockwave therapy is widely applied in musculoskeletal disorder treatment (Tamma et al. 2009). Over the last 10 years, this therapy has been used in other fields of medicine, such as treatment of impaired wound healing, burn injuries (Novak et al. 2008; Sathishkumar et al. 2008; Haupt 1997), and erectile dysfunction (Vardi et al. 2012). The recent transformation in the application of shock waves for the stimulation of the healing process has become possible due to the understanding of the effects of shock waves on neovascularization, differentiation of stem cells into injured tissue cells, and the release of different growth factors (Sathishkumar et al. 2008; Nishida et al. 2004; Wang et al. 2003). Recently, the anti-inflammatory effect of shockwave therapy has also been reported (Mittermayr et al. 2012; Mariotto et al. 2009). Although the biochemical mechanisms underlying this effect are not fully understood, extracorporeal shockwave therapy (ESWT) may modulate endogenous nitric oxide (NO) production by positively affecting endothelial NO synthase activity and subsequently suppressing NF-κB activation (Mariotto et al. 2009). In addition, the level of macrophage-derived inflammatory proteins (MIP-1α and MIP-1β) and the oxidative burst of leukocytes were reduced after ESWT (Mittermayr et al. 2012).

Orthodontic tooth movement is an accepted model for inducing and resolving inflammation during a limited time period (Proffit 2006). The prolonged pressure applied by the orthodontic appliance on a tooth results in its movement due to a weakening of the periodontal ligament (PDL), a collagenous supporting structure, as the bone around the tooth remodels. Like any inflammatory reaction, it involves two phases. An acute phase (the first 1–3 days) is initiated by resident macrophages that undergo activation and release inflammatory mediators enabling propagation and maturation of the inflammatory response (Eugene Roberts 2005). When stimulation (i.e., the orthodontic pressure) is sustained, a shift occurs toward the chronic phase, which is characterized by tissue destruction and resolution due to persisting inflammatory process.

Rat models of tooth movement have provided *in vivo* evidence that the receptor activator of nuclear factor kappa-B ligand (RANKL), interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), and IL-6 are up-regulated in PDL cells and osteoblasts (Hazan-Molina et al. 2013; Kim et al. 2007; Bletsa et al. 2006; Alhashimi et al. 2001; Shiotani et al. 2001), and are important regulators of bone remodeling in response to mechanical stimulation (Yamaguchi 2009; Ren et al. 2007; Kanzaki et al. 2006; Uematsu et al. 1996; Le and Vilcek 1987). These cytokines are elevated in the gingival crevicular fluid of patients during orthodontic treatment within a few days after force application (Nishijima et al. 2006; Lowney et al. 1995; Grieve et al. 1994).

In our previous preliminary study, the application of shock waves during orthodontic tooth movement increased acutely the expression of IL-1β in the gingival crevicular fluid on the compressed side of the PDL on Day 2, along with a reduction in the number of TRAP (tartrate resistant acid phosphatase) positive cells, characteristic of osteoclasts on Day 3. These findings contradicted each other, since IL-1β is known to promote osteoclastogenesis by inducing RANKL (receptor activator of nuclear factor κβ ligand) expression on stromal cells and synergizes with RANKL to promote osteoclast differentiation (Bloemen et al. 2011; Lee et al. 2010; Takayanagi 2007; Teitelbaum 2006). In an attempt to resolve the controversy, in the present study we set out to further investigated the expression of acute phase cytokines in the rat periodontal tissue using an alternative method of cytokine determination. The study was conducted in two separate sets of experiments. In the first set, ESWT was compared to a sham control (no orthodontic force). In the second set, orthodontic force was applied with and without ESWT. Both sets of experiments lasted for 3 days; the time that covers the acute phase of the orthodontic inflammatory reaction.

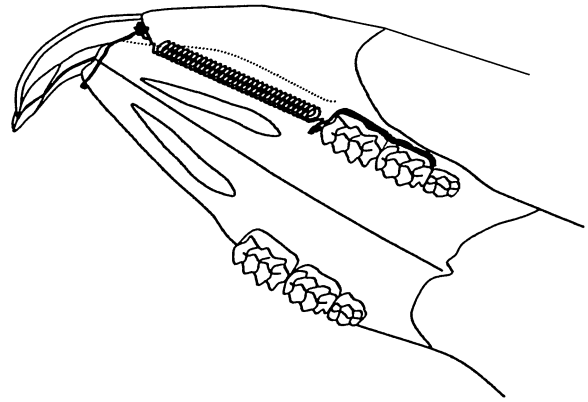

Fig. 1 Scheme of the orthodontic rat model

2 Methods

All procedures were approved by the Institutional Animal Care and Use Committee at the Technion – Israel Institute of Technology (IL-0005-01-11). Twenty one, 3–4 months old, Wistar male rats, weighing 260–280 g were used in this study. The animals were fed a standard pellet diet and water ad libitum, and kept at 25 °C (±2 °C) while alternating 12-h periods of light and dark. Following acclimation (1 week), rats were randomly categorized into 4 groups: without an orthodontic appliance or ESWT (negative control: n = 3); ESWT without an orthodontic appliance (ESWT: n = 6); with an orthodontic appliance and without ESWT (Spring: n = 6); and with an orthodontic appliance and ESWT (Spring + ESWT: n = 6). General anesthesia using 75 mg/kg of ketamine (Rotexmedica; Trittau, Germany) and 10 mg/kg of xylazine (Eurovet Animal Health B.V.; Bladel, the Netherlands), administered by intramuscular injection in a hindlimb, was induced for the application of the orthodontic appliance and for the shock wave therapy.

2.1 The Orthodontic System

In each rat, the experimental side was randomly chosen and the skin above it was shaved. A stainless steel ligature wire (0.012″) (SIA; Orthodontic Manufacturer, Caserta, Italy) was bent and inserted beneath the contact point of the second and third maxillary molars, thus enclosing the first and second maxillary molars as a single unit on each experimental side. The contralateral side was not used as control due to short distance from the experimental side and the possible effect of the ESWT. A 20 cN sentalloy closed coil spring (GAC; Central Islip, NY) was attached to this ligature wire and tightened to the teeth (Fig. 1). A transverse hole was drilled through both maxillary incisors at the apical third of the crown using a drilling bur and the stainless steel ligature wire was inserted through the hole as previously described (Ren et al. 2004). When the pulp exposure occurred, dentine continued to build up thus forming a bridge over the exposure site and sealing it during the following 3 days of the study (Inoue and Shimono 1992). The Sentalloy® spring was activated and subsequently attached to the ligature wire through the incisors. The spring's delivered force was tested and confirmed to produce a force of 20 ± 2 cN. There was no reactivation during the experimental period.

2.2 ESWT Application

Based on the literature reports (Sathishkumar et al. 2008; Nishida et al. 2004), right after surgical application of the orthodontic appliance, the rats from the ESWT and Spring + ESWT groups were treated with a single application of 1000 unfocused impulses at EFD 0.1 mJ/mm^2, with a

pulse rate of 5 pulses per sec by DermaGold (MTS; Konstanz, Germany) to the area of the maxillary tuberosity, i.e., the anatomical location of the 3 maxillary molars.

2.3 Gingival Crevicular Fluid (GCF) Collection

GCF was collected under general anesthesia during the 3 days of the experiment. The upper second molar, on the experimental side, was isolated with cotton rolls and gently dried with an air syringe. Paper filter strips (Periopaper – gingival fluid collection strips; Pro Flow; Amityville, NY) were longitudinally cut, carefully inserted into the gingival crevice until mild resistance was felt and remained there for 30 s. Upon removal, the paper strips were covered with aluminum foil, placed in an Eppendorf tube and stored at −20 °C. Paper filter strips contaminated with saliva or blood were placed aside and reapplied to the gingival crevice after 1 h. This method has been used to collect and analyze small volumes of biological fluids (Perinetti 2004). Sampling of GCF often collects the entire volume of fluid at the sampled site, and this volume varies from tooth site to tooth site. As a result, an approach to GCF sampling was developed, which standardizes the time of collection and reports the data as a total amount (or activity) in the timed sample (Lamster et al. 1998; Lamster 1997).

2.4 Determination of Cytokine Concentrations

Cytokine concentrations (pg/ml) were determined using a commercial 4-multiplex fluorescent bead-based immunoassay (Procarta Cytokine Assay Kit; Affymetrix, Santa Clara, CA) and the Luminex 100 IS Instrument (Luminex; Austin, TX). The multiplex kit used was capable of detecting pro-inflammatory cytokines: IL-1β, IL-6, soluble receptor activator of nuclear factor kappa-B ligand (sRANKL) and tumor necrosis factor (TNF)-α.

For the assay, each paper strip was inserted into an individual test tube containing 75 μL of PBS. Tubes were kept at room temperature for 30 min, and were shaken every 5 min to facilitate extraction of the sample from the filter papers. After eluting the gingival crevicular fluid from the paper strips by centrifugation at 14,000 × g for 5 min, 50 μL aliquots/well of the gingival crevicular fluid samples were incubated with anti-rat multi-cytokine beads at room temperature for 1 h in the dark. The unbound material was removed by filtration. Twenty-five microliters/well of detection antibodies were added, and reactions were incubated at room temperature for 30 min in the dark. Twenty-five microliters/well of streptavidin–phycoerythrin were then added, and the plates were incubated at room temperature for an additional 30 min in the dark. One hundred and twenty microliters/well of reading buffer were added and the plate was read in a plate reader. Concentrations of cytokines in each sample were extrapolated from standards by means of Luminex 100 Integrated System 2.3 software (Austin, TX). All samples were run in duplicates.

2.5 Statistical Analysis

Results were expressed as means ± SE. The inter-group differences in IL-1β/IL-6/sRANKL/TNF-α levels in the multiplex fluorescent bead-based immunoassay were assessed with the Kruskal-Wallis test on ranks, and multiple comparisons were adjusted by the Mann-Whitney U test with Holm's sequential Bonferroni correction. The differences in the cytokine levels between the 3 days of the experiment were assessed by the Wilcoxon signed-rank test. Statistical significance was assumed to be at $p < 0.05$. Data were evaluated using SPSS software, ver. 17 (SPSS, IBM, Chicago, IL).

3 Results

All cytokines displayed a similar trend in both the Spring + ESWT and Spring groups, whereby

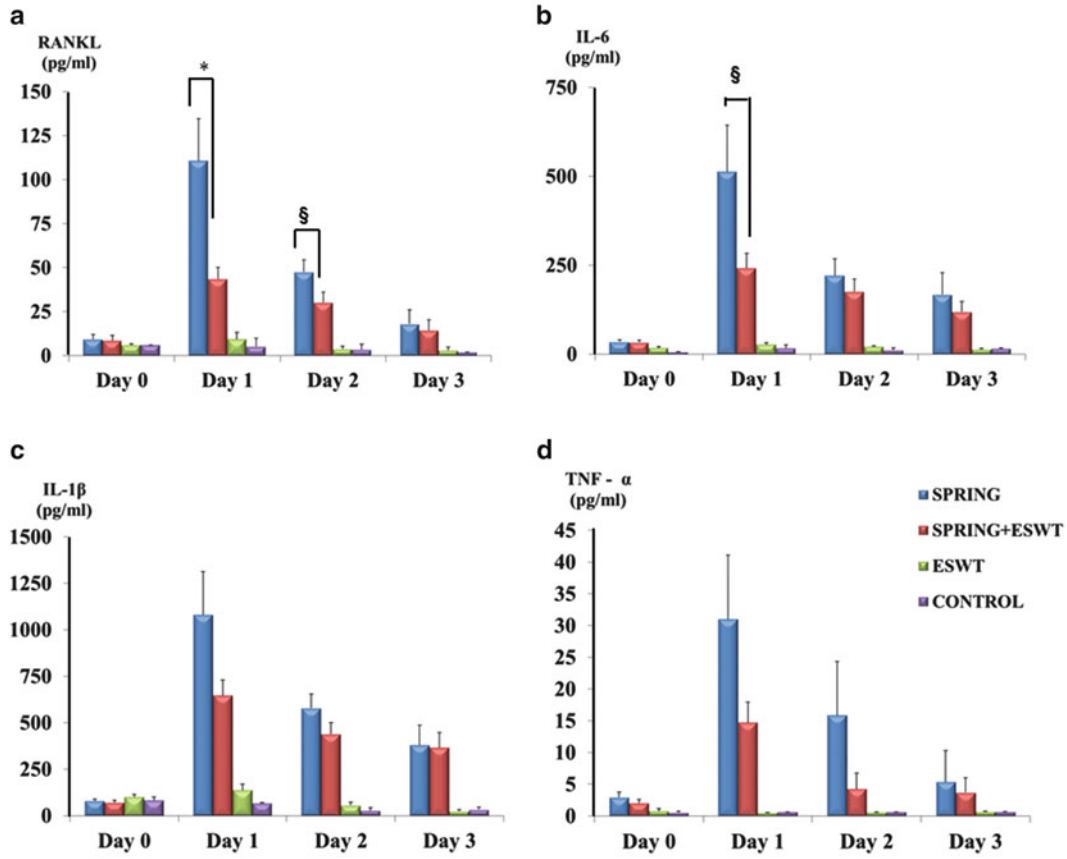

Fig. 2 Concentration of different cytokines in the four study groups during a 3-day study. (**a**) – sRANKL; (**b**) – IL-6; (**c**) – IL-1β; (**d**) – TNF-α. Data are means ± SE. *p = 0.01; §p = 0.07

the concentration peaked on Day 1 and decreased thereafter (Fig. 2). In all cases, the level of cytokines was smaller in the Spring + ESWT than that in the Spring group. However, a statistical significance was only revealed in sRANKL concentration ($p = 0.01$) and a borderline significance in the IL-6 concentration ($p = 0.07$). On Day 2, the difference in cytokine concentrations between the two groups decreased, with sRANKL in the Spring + ESWT group maintaining a borderline lower level compared with the Spring group ($p = 0.07$) (Fig. 2). On Day 3, all differences in cytokine concentrations between the two groups levelled off.

The ESWT and negative control groups also displayed a similar trend with no statistically significant difference found between the two in regard to all cytokines during the 3 days of the study (Fig. 2).

We also examined the change in each cytokine concentration during the 3 days in each group. In the Spring + ESWT and Spring groups, increases in all cytokines were significant between Days 0 and 1 ($p \leq 0.01$). Then, on Day 2 the level of cytokines reverted in both groups, but the reversion was significant only regarding sRANKL and IL-1β ($p \leq 0.05$).

In the negative control group, all cytokines remained quite stable and their change was largely insignificant between the days of the experiment (Fig. 2). Nevertheless, in the ESWT group, sRANKL displayed a significant increase from Day 0 to Day 1 ($p = 0.04$), and IL-1β and IL-6 demonstrated a significant decrease from Day 1 to Day 2 ($p < 0.05$).

4 Discussion

In the present study we determined the effects of extracorporeal (ESWT) shockwave therapy on the expression of cytokines in the periodontal tissues after induction of orthodontic force. The force of 20 cN was distributed over the two first maxillary molars. Since the human molar is approximately 20 times larger than the rat molar (Sato et al. 1984), the effect of force could be estimated to be the same as the force of 200 cN on a human molar, which is considered a substantial orthodontic force (Lee et al. 2013).

We selected the cytokines for the study which are known to respond with increases following orthodontic force activation (Uematsu et al. 1996; Lowney et al. 1995; Grieve et al. 1994) to assess the effect of ESWT on the inflammatory process in general and on its initiation in particular. The evidence of similar pattern of biologic reactions in the presence of ESWT indicates that this treatment leads to a modification of the inflammatory process rather than to its elimination. Spring activation, as a means of achieving orthodontic tooth movement, created an inflammatory response expressed by increases in the concentration of all cytokines. The increases peaked 24 h after the application of force application, which is in accord with previous reports (Karacay et al. 2007; Ren et al. 2007; Dudic et al. 2006; Nishijima et al. 2006; Tian et al. 2006). The anti-inflammatory effect of ESWT, previously reported by Mariotto et al. (2009), was validated in this study as all cytokines were reduced in the GCF after shockwave therapy. Moreover, a significant decrease in the concentration of sRANKL after ESWT may indicate a positive effect exerted by ESWT on bone formation. That is also supported by Tamma et al. (2009) who have reported a decrease in the ratio of sRANKL to osteoprotegerin after ESWT, suggesting the inhibition of osteoclastogenesis.

Several studies that have described a specific effect of ESWT in different cell cultures consisting of an elevation of different cytokines (Nishida et al. 2004; Han et al. 2009; Hofmann et al. 2008). In the present *in vivo* study, concentration of all cytokines in both ESWT and negative control groups did not differ significantly on all study days. Application of ESWT in *in vivo* model should be followed by induction of inflammatory processes, such as flap creation (Yan et al. 2008), ischemia-induced myocardial dysfunction (Nishida et al. 2004), periodontal disease induction (Sathishkumar et al. 2008), or spring activation during orthodontic force application to detect changes in cytokine concentration. ESWT alone is probably insufficient to cause an appreciable change in the tissue cytokine profile.

In our previous study (Hazan-Molina et al. 2013), we have observed a significantly higher concentration of IL-1β in the GCF on Day 2 in the Spring + ESWT group, along with a reduced number of TRAP positive cells, a constituent of osteoclasts, on the compressed side of PDL on Day 3, compared with the Spring alone group. IL-1β promotes osteoclastogenesis by inducing RANKL expression in stromal cells and synergizes with RANKL to promote osteoclast differentiation later on (Bloemen et al. 2011; Lee et al. 2010; Takayanagi 2007; Teitelbaum 2006). IL-6 is also known as a potential osteoporotic factor due to its effect on osteoblast lineage cells (Chung et al. 2003; Ota et al. 2001). Thus, we could expect a similar pattern of expression of IL-1β, IL-6, and RANKL in the GCF during the study days. The present findings regarding IL-1β, IL-6, and RANKL in GCF were generally in line with the previous reports above mentioned and with our expectations, although only a trend was noted in some cases. Furthermore, Han et al. (2009) detected a non-statistically significant decrease in IL-1β concentration, along with a statistically significant decrease in IL-6. This divergent effect, compared with our previous studies (Hazan-Molina et al. 2013; Hazan-Molina et al. 2011), might be explained by the use of different cytokine detection methods, i.e., ELISA and 4-multiplex fluorescent bead-based immunoassay in the previous and in this study, respectively. The inability to detect other than IL-1β cytokines in the GCF in the previous study and

the contradiction in the expression pattern between IL-1β and TRAP positive cells led us to search for a different detection method more suitable for small volumes. Multiplex fluorescent bead-based immunoassay has been known as a very sensitive and specific method for the detection and quantitation of different proteins in human sera and other body fluids of small volumes (Martins et al. 2004; Kellar et al. 2001). The ability to consistently repeat the pattern of expression of all cytokines in the present study may indicate that the 4-multiplex fluorescent bead-based immunoassay proved superior to ELISA in cytokine detection in GCF.

It is likely that despite some anti-inflammatory effect of ESWT, the initial inflammatory reaction may be strong enough to propagate into an orthodontic tooth movement. Furthermore, since a faster healing process is expected, due to a smaller inflammatory reaction, paradoxically an even larger orthodontic tooth movement could be anticipated with ESWT for the same orthodontic force applied during the same time frame.

The surprising results presented in this study raise a question of whether the anti-inflammatory effect of ESWT may have a clinical potential to be implemented in enhancing orthodontic tooth movement. This study focused on the first 3 days of the inflammatory reaction. During this time frame the tooth is only minimally displaced in the alveolar socket. Further research should be conducted through a longer time frame (at least 3 weeks), comparing the amount of orthodontic tooth movement with and without the effect of ESWT. In addition, ESWT may be also implemented in the future for the treatment of different lesions, involving acute inflammatory reactions in the oral cavity with damage to the alveolar bone, due to the ability of ESWT to reduce the inflammatory process and inhibit osteoclastogenesis. One example is periodontal diseases that often leads to osseous defect and teeth loss or periodontal inflammation around implants (peri-implantitis).

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

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Thyroid Hormone Levels and TSH Activity in Patients with Obstructive Sleep Apnea Syndrome

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Abstract

Obstructive sleep apnea syndrome (OSAS) is characterized by complete cessation of inspiratory flow (apnea) or upper airway airflow limitation (hypopnea) with increased respiratory muscle activity, which is repeatedly observed during sleep. Hypothyroidism has been described as a rare cause of OSAS, but it is considered to be the main cause of breathing disorders during sleep in patients in whom an improvement of OSAS is observed after thyroid hormone replacement therapy. Nevertheless, euthyreosis due to thyroxine replacement in patients with OSAS often does not improve the breathing disorder and treatment with continuous positive airway pressure is usually applied. The aim of this study was to assess thyroid function in patients with OSAS. We studied 813 patients in whom severe OSAS was diagnosed; the mean apnea-hypopnea index was 44.0. Most of the patients were obese (mean BMI 33.1 $\pm$ 6.6 kg/m^2) and had excessive daytime sleepiness (ESS 12.8 $\pm$ 6.6). With the thyroid stimulating hormone (TSH) concentration as the major criterion, hypothyroidism was diagnosed in 38 (4.7 %) and hyperthyroidism was diagnosed in 31 (3.8 %) patients. Analysis of basic anthropometric data, selected polysomnography results, and TSH, fT3, and fT4 values did not reveal any significant correlations. In conclusion, the incidence of thyroid function disorders seems to be no different in OSAS than that in the general population. We did not find correlations between TSH activity and the severity of breathing disorders during sleep.

Keywords

fT3 • fT4 • Hyperthyroidism • Hypothyroidism • OSAS • TSH

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1 Introduction

Obstructive sleep apnea syndrome (OSAS) is characterized by complete cessation of inspiratory flow (apnea) or upper airway airflow

limitation (hypopnea) with increased respiratory muscle activity, which is repeatedly observed during sleep. OSAS is the most frequent disease among breathing disorders during sleep. Prevalence of OSAS ranges from 4 to 10 % of middle-aged men and from 2 to 4 % of middle-aged women (Young et al. 1993; Plywaczewski et al. 2008). The most important risk factors for OSAS are obesity, craniofacial structural abnormalities (curvature of the nasal septum, hyperglossia, structural abnormalities and position of the mandible, overgrown tonsils, and structural abnormalities of the soft palate and the uvula), and a decreased upper airway muscle activity.

Hypothyroidism has been described as a rare cause of OSAS. In 1964, Massumi and Winnacker (1964) reported a case of myxoedema and apneas. Hypothyroidism is considered to be the main cause of breathing disorders during sleep in patients in whom an improvement of OSAS is observed after thyroid hormone replacement therapy (Orr et al. 1981; Skatrud et al. 1981; Millman et al. 1983). However, euthyreosis due to thyroxine replacement in patients with OSAS often does not improve the breathing disorder and in these patients CPAP treatment is usually applied. According to Grunstein and Sullivan (1988), only does combined treatment significantly reduce the risk of myocardial infarction in these patients.

The incidence of OSAS in patients with hypothyroidism has been reported as high as 80 % (Grunstein and Sullivan 1988; Rajagopal et al. 1984). However, some of the later publications do not demonstrate a significant correlation between low thyroid hormone activity and the incidence or severity of OSAS (Resta et al. 2005). Lin et al. (1992) and Pelttari et al. (1994) have found a lower incidence hypothyroidism and OSAS. Winkelman et al. (1996) have found low thyroid hormone activity only in 3 of 103 patients with OSAS and concluded that in the absence of clinical symptoms consistent with hypothyroidism, OSAS patients do not require routine assessment of serum TSH and thyroxine levels. Similar results were obtained by Kapur et al. (1998) who noted hypothyroidism in 4 out of 284 patients with breathing disorders during sleep. Taking into account these equivocal reports, we decided to assess the thyroid hormonal status in a large unselected group of OSAS patients in order to establish a rationale for the routine thyroid function testing in this population.

2 Methods

The study was approved by the institutional Review Board for Human Research. This retrospective study included 813 patients (F/M – 146/667). The diagnosis of OSAS was established in accordance with the American Academy of Sleep Medicine recommendations (AASM 2014). All patients underwent a 7-h polysomnography (Embla System – S4000; Reyjkjavik, Iceland) during which airflow, chest and abdominal motion, and the oxygen arterial blood saturation were monitored. Airflow was measured with the use of a pressure cannula or a three-channel thermistor placed in the front nostrils and the mouth area. Chest and abdominal motion were recorded with the use of two piezoelectric belts placed around the abdomen and chest; an integral part of the polysomnography system. Oxygen arterial blood saturation was recorded with pulse oximetry. The electroencephalogram (EEG), electrooculogram (EOG) and electromyogram (EMG) were recorded to determine sleep structure and number of awakenings. During sleep cardiac function was monitored by a one-lead ECG. The Epworth Sleepiness Scale (ESS) was used to assess the daytime sleepiness (Johns 1991). The diagnosis of OSAS was made when the apnea-hypopnea index (AHI) value exceeded 5.0.

Fasting serum concentrations of thyroid stimulating hormone (TSH), free triiodothyronine (fT3) and free thyroxine (fT4) were assessed by electrochemiluminescence (Roche Diagnostics; Basel, Switzerland). The normal value for our laboratory ranges from 0.27 to 4.2 μIU/ml for TSH, 3.1–6.8 pmol/l for fT3, and 12.0–22.0 pmol/l for fT4.

Data are reported as means ± SD. To assess the influence of TSH, fT3, and fT4 on the polysomnography results the non-parametric Mann-Whitney U test was applied. Statistical analysis was performed with Statistica 9 Software (StatSoft; Tulsa, OK).

3 Results

We studied 813 patients in whom severe OSAS was diagnosed; the mean AHI was 44.0. Most of the patients were obese (mean BMI 33.1 ± 6.6 kg/m^2) and had excessive daytime sleepiness (ESS, 12.8 ± 6.6). Table 1 presents the characteristics of the group and the polysomnography (PSG) results.

Lowered thyroid function was noted in 38 patients. Three patients from this group had been previously diagnosed and received replacement treatment with thyroxine. Thirty one patients had laboratory features of hyperthyroidism. Table 2 presents the characteristics of those groups.

With the TSH concentration as the major criterion, hypothyroidism was diagnosed in 38 (4.7 %) and hyperthyroidism was diagnosed in 31 (3.8 %) patients, respectively. Analysis of the basic anthropometric data, selected PSG results, and TSH, fT3 and fT4 values did not reveal significant correlations.

4 Discussion

Hypofunction of the thyroid gland in patients with OSAS has been reported in the literature. Some authors report the incidence of hypothyroidism in patients with OSAS to be as high as 40 % (Hira and Sibal 1999). Hypothyroidism is often accompanied by obesity, a major risk factor for OSAS. On the other hand, replacement therapy with thyroxine results in a decrease in the number of apneas even without a reduction in body weight, indicating a more complex

Table 1 Anthropometric characteristics, selected polysomnography parameters and thyroid hormone activity in 813 OSAS patients enrolled into the study

Age (yr)	54.3 ± 10.3
BMI (kg/m^2)	33.1 ± 6.6
Epworth sleepiness scale	12.8 ± 5.9
AHI (events/h)	43.9 ± 21.2
HI (events/h)	17.4 ± 13.7
Minimal SpO_2 (%)	67.9 ± 13.4
Mean SpO_2 (%)	89.6 ± 5.3
Total time with SpO_2 < 90 % (min)	107 ± 108
TSH (μIU/ml)	1.9 ± 4.0
fT3 (pmol/l)	4.6 ± 1.2
fT4 (pmol/l)	13.6 ± 3.3

Data are means ± SD. *BMI* body mass index, *AHI* - apnea-hypopnea index, *HI* hypopnea episodes, *SpO_2* arterial blood oxygen saturation in pulse oximetry, *TSH* thyreotropin, *fT3* free 3-ioido-thyronine, *fT4* free thyroxine

Table 2 Anthropometric characteristics, selected polysomnography parameters and thyroid hormone activity in OSAS patients with hypothyroidism and hyperthyroidism; data are means ± SD

	Patients with hypothyroidism	Patient with hyperthyroidism
Age (yr)	56.4 ± 11.9	55.3 ± 10.2
BMI (kg/m^2)	31.9 ± 6.8	35.3 ± 6.1
Epworth sleepiness scale	11.4 ± 5.9	12.1 ± 5.9
AHI (events/h)	47.1 ± 23.9	40.4 ± 23.5
HI (events/h)	19.2 ± 12.3	21.2 ± 13.8
Minimal SpO_2 (%)	68.3 ± 12.4	66.8 ± 13.3
Mean SpO_2 (%)	89.9 ± 4.6	87.9 ± 5.3
Total time with SpO_2 < 90 % (min)	116 ± 88	153 ± 108
TSH (μIU/ml)	12.8 ± 14.6	0.1 ± 0.1
fT3 (pmol/l)	4.3 ± 2.1	4.7 ± 1.2
fT4 (pmol/l)	9.4 ± 5.6	14.1 ± 3.0

Data are means ± SD. *BMI* body mass index, *AHI* apnea-hypopnea index, *HI* hypopnea episodes, *SpO_2* arterial blood oxygen saturation in pulse oximetry, *TSH* thyreotropin, *fT3* free 3-ioidothyronine, *fT4* free thyroxine

mechanism of breathing disorders in these patients (Rajagopal et al. 1984). Some authors hypothesize that OSAS may also be a result of hypothyroid myopathy leading to upper airway muscle collapse and narrowing during sleep (Kendall-Taylor and Turnbull 1983). Thyroid hormone replacement therapy does not always result in a reduction of breathing disorders. In the series of patients studied by Misiolek et al. (2007), patients receiving replacement therapy showed a lower snoring intensity, but the respiratory disturbance index (RDI) did not change significantly; neither did the minimal oxygen saturation during sleep.

The accumulation of mucopolysaccharides in the upper airways, particularly in the throat and the palate, may be yet another pathogenic factor (Orr et al. 1981). In the study by Jha et al. (2006), hyperglossia was diagnosed in 33 % of the patients with hypothyroidism, and edema of the face or throat was noted in 83 %.

The prevalence of hypothyroidism in the patients with OSAS of the present study was 4.7 % and this is comparable with the incidence of hypothyroidism in adult population of Poland, estimated to be 2–5 % (Zgliczynska 1998). One study has reported a much lower prevalence of hypothyroidism in OSAS at 1.5 %; however, it included a smaller group consisting of 103 OSAS patients (Winkelman et al. 1996).

The present results showed no significant correlation between the TSH activity and the severity of OSAS; the correlation between TSH and age is well known and has also been confirmed in this study. TSH abnormalities alone are not necessarily associated with higher AHI/RDI values, as it has been shown that mainly the fT3 and fT4 concentrations influence breathing in a hypothyroid patient. These observations have been confirmed by Winkelman et al. (1996) who demonstrate that a higher TSH value indicates a higher risk of OSAS only in women over 60 years of age. One may assume that fT3 and fT4 concentrations are better markers in predicting apnea risk. Lower daytime sleepiness and higher oxygen saturation may be ameliorated by these two hormones, and we may hypothesize that they can relieve apnea symptoms.

The present study demonstrates that there is no rationale for a routine assessment of thyroid activity in patients suspected of breathing disorders during sleep. We agree with Winkelman et al. (1996) that thyroid hormone testing should be limited to patients with clinical symptoms suggestive of hypothyroidism such as characteristic skin color, psychomotor retardation, excessive sleepiness and asthenia, or a large increase in body weight. It should be remembered, however, that Deegan et al. (1997) have described a patient with biochemical features of hypothyroidism, without clinical symptoms, in whom significant breathing disorders during sleep were found. Therefore, assessment of thyroid hormones should be individually considered. In conclusion, the incidence of thyroid function disorders in sleep apnea patients seems to be no different than that in the general population. We failed to find correlations between TSH activity and the severity of breathing disorders during sleep.

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

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Age and Gender-Related Changes in Biogenic Amine Metabolites in Cerebrospinal Fluid in Children

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Abstract

Metabolites of cerebrospinal biogenic amines (dopamine and serotonin) are an important tool in clinical research and diagnosis of children with neurotransmitter disorders. In this article we focused on finding relationships between the concentration of biogenic amine metabolites, age, and gender. We analyzed 148 samples from children with drug resistant seizures of unknown etiology and children with mild stable encephalopathy aged 0–18 years. A normal profile of biogenic amines was found in 107 children and those children were enrolled to the study group. The CSF samples were analyzed by HPLC with an electrochemical detector. The concentrations of the dopamine and serotonin metabolites homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA), respectively, were high at birth, gradually decreasing afterward until the 18 years of age. Nevertheless, the HVA/5-HIAA ratio did not vary with

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age, except in the children below 1 year of age. In the youngest group we observed a strong relationship between the HVA/5-HIAA ratio and age ($r = 0.69$, $p < 0.001$). There were no statistical differences in the level of both dopamine and serotonin metabolites between boys and girls, although a trend toward lower HVA and 5-HIAA in the boys was noticeable. Significant inter-gender differences in the level of HVA and 5-HIAA were noted only in the age-group of 1–4 years, with 5-HIAA being higher in the girls than boys ($p = 0.004$). In conclusion, the study revealed that the concentration of biogenic amine metabolites is age and sex dependent.

Keywords
Biogenic amines • Brain metabolism • Cerebrospinal fluid • Children • Gender • Neurotransmitters

1 Introduction

Neurotransmitters play a central role in the brain metabolism as they modulate electrical signals between neurons. Neurotransmitters are synthesized and stored in the presynaptic nerve terminals and are released on stimulation into the synaptic cleft, acting on postsynaptic membrane receptor sites. The mechanisms controlling the different stages of this process are diverse and complex and are designed to ensure the balance of neurotransmission. An array of neurotransmitters should be functionally integrated to facilitate the enormous dynamics within the nervous system. Disorders that affect the neurotransmitter metabolism, transport, and storage are complex and may deeply impair neurological functions (Hyland 1999; Kandel et al. 2000).

A variety of neurotransmitters fall into various chemical classes, such as biogenic amines (catecholamines and serotonin), amino acids (γ-amino butyric acid (GABA), or glutamic acid and glycine). Disorders of these neurotransmitters are due to their aberrant metabolism, transport or defects of cofactors: active form of pirydoxine (pyridoxine-5-phosphate, PLP) and tetrahydrobiopterin (BH_4) (Hoffmann et al. 1998).

Biogenic amines (BA) play key roles in both central and autonomous nervous systems having a diverse range of action. They provide control of psychomotor function; regulate vascular tone, and body temperature. Serotonin plays an essential role in the control of emotional stability, memory, appetite, mood, and sleep. Dopamine and serotonin are synthesized from amino acid precursors: tyrosine and tryptophan, respectively. The first step in the synthesis is hydroxylation of tyrosine and tryptophan by BH_4-depending hydroxylases to L-dopa and 5-OH-tryptophan. Then, L-dopa and 5-OH-tryptophan are further decarboxylated to dopamine and serotonin, respectively, by aromatic amino acid decarboxylase (AADC) (Hyland et al. 1993; Van der Heyden et al. 2003).

BH_4 is a cofactor for phenylalanine, tyrosine, and tryptophan hydroxylases. It is oxidized to its hydroxyl derivative pterin-4α-carbinolamine during the hydroxylation process. Pterin-4-α-carbinolamine, in turn, is converted by pterin-4-α-carbinolamine dehydratase (PCD) to quininoid dihydrobiopterin (qBH_2). The next step is regeneration of BH_4, were qBH_2 is converted to BH_4 by dihydropteridine reductase (DHPR). BH_4 is synthesized from GTP in the four steps catalyzed by GTP cyclohydrolase I (GTPCH I), 6-pyruvoyl-tetrahydropterin synthase (PTPS), and sepiapterin reductase (SR) (Blau et al. 2001).

The clinical diagnosis of biogenic amine metabolism disorders is challenging and needs detailed biochemical analysis including

cerebrospinal dopamine and serotonin metabolites, homovanilic acid (HVA) and 5-hydroxyindoloacetic acid (5-HIAA), respectively. Additionally, clinically relevant metabolite of L-dopa is 3-orto-methyl-dopa (3-OMD). Cerebrospinal fluid (CSF) analysis and interpretation of results is difficult since many factors may influence the levels of monoamine metabolites, such as patient's age, body height, diet, motility, and site of puncture and position of the lumbar puncture needle (Kurian et al. 2011). Another well-known confounding factor is the presence of concentration gradients in the CSF (Hyland 2006). Therefore, the lumbar puncture and CSF collection has to be performed according to a detailed protocol. Hyland et al. (1993) and Kurian et al. (2011) have described the occurrence of a diurnal gradient variation of neurotransmitter metabolites. The analysis of BH_4 and biogenic amine metabolites requires the same CSF fractions for all analyzed metabolites. Concentration of dopamine and serotonin metabolites is the highest at birth, and then declines in the first few months of life. A high concentration of HVA and 5-HIAA in neonates reflects active mitosis, neurogenesis, migration, and formation of neuronal networks in the fetal and neonatal brains (Kurian et al. 2011).

It is widely accepted that neurotransmitters display variable patterns across gender and age reflecting developmental and maturation processes of the central nervous system. As such, concentrations of biogenic amine metabolites are expected to differ between the neonatal period, early childhood, prepubertal years, puberty, and adulthood (Blau et al. 2005). In addition, differences may be also found between boys and girls. The only available data on the gender difference have been described in adults. Therefore, it is essential to establish pediatric reference data reflecting these changes (i.e., stratified according to gender, age, and developmental stage) to aid in differentiating between children with and without inherited neurometabolic diseases (Van der Heyden et al. 2003; Kusmierska et al. 2009).

The aim of the current study was to demonstrate in children an interconnection of gender and age with drug resistant seizures of unknown etiology and with mild stable encephalopathy (mainly cerebral palsy), both unrelated to inherited disorders of biogenic amines, and to characterize the pattern of monoamine metabolites in CSF from infancy to early adulthood in both girls and boys.

2 Methods

In the period of 2008–2011 biogenic amine metabolites were assessed in CSF samples from 68 children with drug-resistant epilepsy and 80 children with non-progressive brain damage of different etiology. Only 107 children with normal level of biogenic amines, pterines and 5-methyltetrahydrofolate (5-MTHF) in CSF were finally enrolled into the study group, including 51 children with drug resistant epilepsy of unknown etiology and 56 children with chronic non-progressive encephalopathy, mainly cerebral palsy (Table 1).

All children eligible for the study were free from any symptoms of extrapyramidal or

Table 1 The number of enrolled children in different subgroups

	Number of children with drug resistant epilepsy	Number of children with chronic non-progressive encephalopathy	
Total	51	56	
Girls	26	32	
Boys	25	24	
Number of children in different age-groups			
Age	0–1 years	1–4 years	>4 years
Girls	15	18	25
Boys	14	15	20

pyramidal syndrome. The enrolled children were recruited from Department of Neurology and Epileptology and Department of Pediatrics, Nutrition and Metabolic Diseases of the Children's Memorial Health Institute, in Warsaw, Poland and from the Neurology Clinic of Mother and Child Institute in Warsaw, Poland. The eligibility criterion was the age of 0–18 years old. In all patients, informed consent from parent or guardian, and child's consent in children older than 16 years was obtained. The study protocol was approved by a local Ethical Committee.

All patients underwent detailed examination using an assessment protocol based on a multidisciplinary approach and administered by an experienced child neurologist. Clinical investigations included: family history and medical records of the child's pre-, and postnatal period. Routine hematology and biochemistry tests including prolactin, glucose, ammonia, lactate, and thyroid function tests in the serum were performed. Neuroimaging tests, including brain magnetic resonance imaging (MRI) and awake and asleep electroencephalograms, were performed in each case. Depending on the clinical picture, biochemical diagnostics included: amino acids in the serum, CSF, and urine, and acylcarnitines in dried blood spot measured by tandem mass spectrometry (MS/MS); urine organic acids analysis by gas chromatography–mass spectroscopy (GC/MS); lysosomal enzymes activities, urinary oligosaccharides and glycosaminoglycans, plasma ceruloplasmin, biogenic amines metabolites, 5-MTHF in CSF (Ormazabal et al. 2005) and pterins profile in CSF and urine (Blau et al. 2005). Patients with well-defined metabolic disorders and storage or degenerative diseases were excluded from the study. In all enrolled patients, the etiological investigations were negative. Considering the onset of epilepsy, CSF samples were analyzed at different time points: during the first month following seizure-onset up to more than 3 years later. All epileptic patients were treated with a combination of anti-epileptic drugs. The drug regiments included sodium valproate, benzodiazepines, topiramate, levetiracetam, phenytoin, oxcarbazepine, and ACTH.

Lumbar puncture following a strict protocol has been applied to obtain CSF sample. Obtained samples were divided into five fractions according to previously described protocol (Kusmierska et al. 2009).

2.1 Analysis of Biogenic Amine Metabolites

The concentrations of HVA and 5-HIAA vary in the different fractions of CSF, therefore only the first portion of 0.5 ml CSF (from the first drops) was used for the analysis. Metabolites of biogenic amines were separated on a reverse phase column (250 × 4.6 × 5). Mobile phase consisted of 10 mM KH_2PO_4, 0.2 mM EDTA, 100 mM octanosulfonic acid, 10 % acetonitrile, and 100 µl TEA (pH 3.0). Directly after thawing, CSF samples were injected into the HPLC (Shimadzu LC-20; Shimadzu Corporation, Kyoto, Japan). Detection was performed by using the electrochemical detector (Antec, DECADE II) at the following parameters: E1 = 590 mV, range – 100 nA, and temperature 35 °C.

Pterin concentration, equal in all fractions of CSF, was analyzed in CSF fraction IV. The total biopterin and neopterin concentration in CSF was measured using HPLC with fluorimetric detection according to the method described by Duarte et al. (2008).

5-MTHF concentration was performed in CSF fraction I according to the procedure described by Verbeek et al. (2008) with own modification.

2.2 Statistical Analysis

Descriptive statistics was used to calculate means ± SD, and minimum-maximum values. Data normality was tested using the Shapiro-Wilk and Kołmogorow-Smirnow test. Comparison of means was carried out using analysis of variance ANOVA. The odds ratio (OR) with 95 % confidence interval (95 % CI) was calculated. A *t*-test

was applied to compare the differences between means in independent assays. The correlation between variables was calculated by Spearman's correlations test. The data were analyzed using Statistica 6 software. A p-value < 0.05 was considered to indicate statistical significant.

3 Results

The HVA and 5-HIAA concentrations in CSF and the interrelationship between the two metabolites in different subgroups were evaluated. Both HVA and 5-HIIA had normal distribution within the examined groups. Concentrations of HVA and 5-HIAA were high at birth and gradually decreased until the age of 18 years; the age-related changes in both monoamine metabolites were significant in the whole study group (HVA: $r = 0.46$; 5-HIAA: $r = 0.57$, $p < 0.0001$ for both). However, either metabolite changed differently throughout the examined life periods. 5-HIAA dropped between the first year of life and 1–4 years of age compared to HVA that did not show a drop (Table 2). However, a rapid decline of HVA was observed in children older than 4 years. The decline rate of HVA between the life periods 0–12 months and 4–18 years was significantly ($p < 0.001$). The minimum-maximum ranges of HVA (0–12 months: 432–633 nmol/l; 1–4 years: 258–825 nmol/l; above 4 years: 118–553 nmol/l), and of 5-HIAA (0–12 months: 192–379 nmol/l; 1–4 years: 119–530 nmol/l; above 4 years: 70–324 nmol/l) were comparable with those reported by other authors in the healthy controls taken as a reference (Heales 2008). There was a significant correlation between HVA and 5-HIAA ($r = 0.44$, $p < 0.001$) (Fig. 1), but no correlation between the HVA/5-HIAA ratio and age in the entire examined group ($r = 0.27$, $p > 0.055$) (Fig. 2). However, a strong relationship between HVA/5-HIAA ratio and age (in months) was found in children below 1 year of age ($r = 0.69$, $p < 0.001$). Although this ratio tended to increase in the successive age-groups, no such strong relationship was seen in either of two groups of children older than 1 year.

There were no significant differences in both monoamines metabolites between boys ($n = 49$) and girls ($n = 58$) across all ages in the entire study group, although a trend was observed toward lower values in boys (Fig. 3a, b). This trend assumed a significantly lower 5-HIAA concentration in boys in the singled out group aged 1–4 years ($p = 0.004$) (Fig. 3c). However, difference in HVA concentration between boys and girls aged 1–4 years was insignificant (Fig. 3d).

Table 2 Biogenic amine metabolites and their ration in the cerebrospinal fluid (CSF) in different age-groups

	n	Mean ± SD	95 % CI
Age 0–12 months	9	5.4 ± 2.6	3.5–7.4
HVA (nmol/l)	9	501.6 ± 70.3	447.5–555.6
5-HIAA (nmol/l)	9	282.3 ± 60.9	235.5–329.2
HVA/5-HIAA	9	1.9 ± 0.5	1.5–2.3
Age 1–4 years	21	25.1 ± 10.8	20.1–30.0
HVA (nmol/l)	21	536.8 ± 152.9	467.2–606.4
5-HIAA (nmol/l)	21	214.5 ± 109.8	164.5–264.4
HVA/5-HIAA	21	2.9 ± 1.3	2.4–3.5
Age > 4 years	21	102.1 ± 45.3	81.5–122.7
HVA (nmol/l)	21	352.2 ± 122.7	296.4–408.1
5-HIAA (nmol/l)	21	125.1 ± 56.6	99.4–150.9
HVA/5-HIAA	21	3.0 ± 1.0	2.5–3.5

Data are means ± SD and 95 % confidence intervals (95 % CI); *HVA* homovanillic acid, *5-HIAA* 5-hydroxyindoleacetic acid

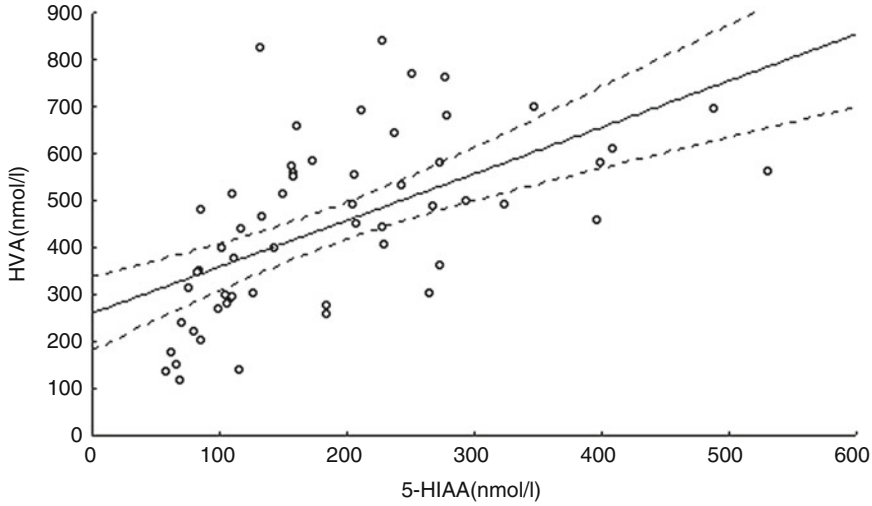

Fig. 1 Correlation between HVA and 5-HIAA in CSF samples

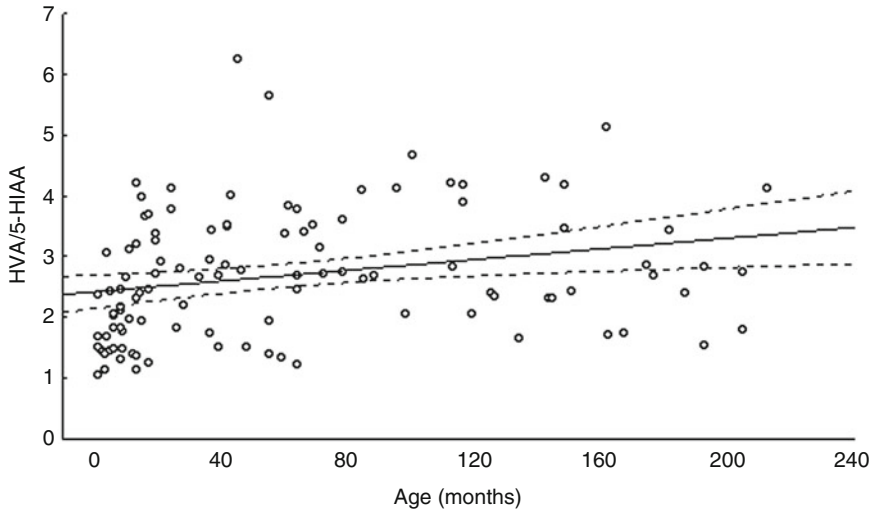

Fig. 2 Lack of correlation between HVA/5-HIAA ratio in CSF samples and child age

4 Discussion

In this report we provide information on monoamine biomarkers in CSF and their distribution by demographic factors (age and sex) in children with drug resistant epilepsy of unknown origin or chronic stable mild encephalopathy. All enrolled patients showed a linear maturation and almost normal development trajectory. In both groups of children, the monoamine metabolites HVA and 5-HIIA remained within the widely accepted, published reference ranges established by Heales (2008). As the ethical issues do not allow subjecting children with no evidence of central nervous system pathology to a painful and invasive procedure of lumbar puncture, we defined a study population that somehow approximates healthy children regarding the biogenic amines metabolism in the brain. The assessment of amine metabolites in CSF reflects their average brain concentration at a single time point. The drawbacks outlined above have a bearing on

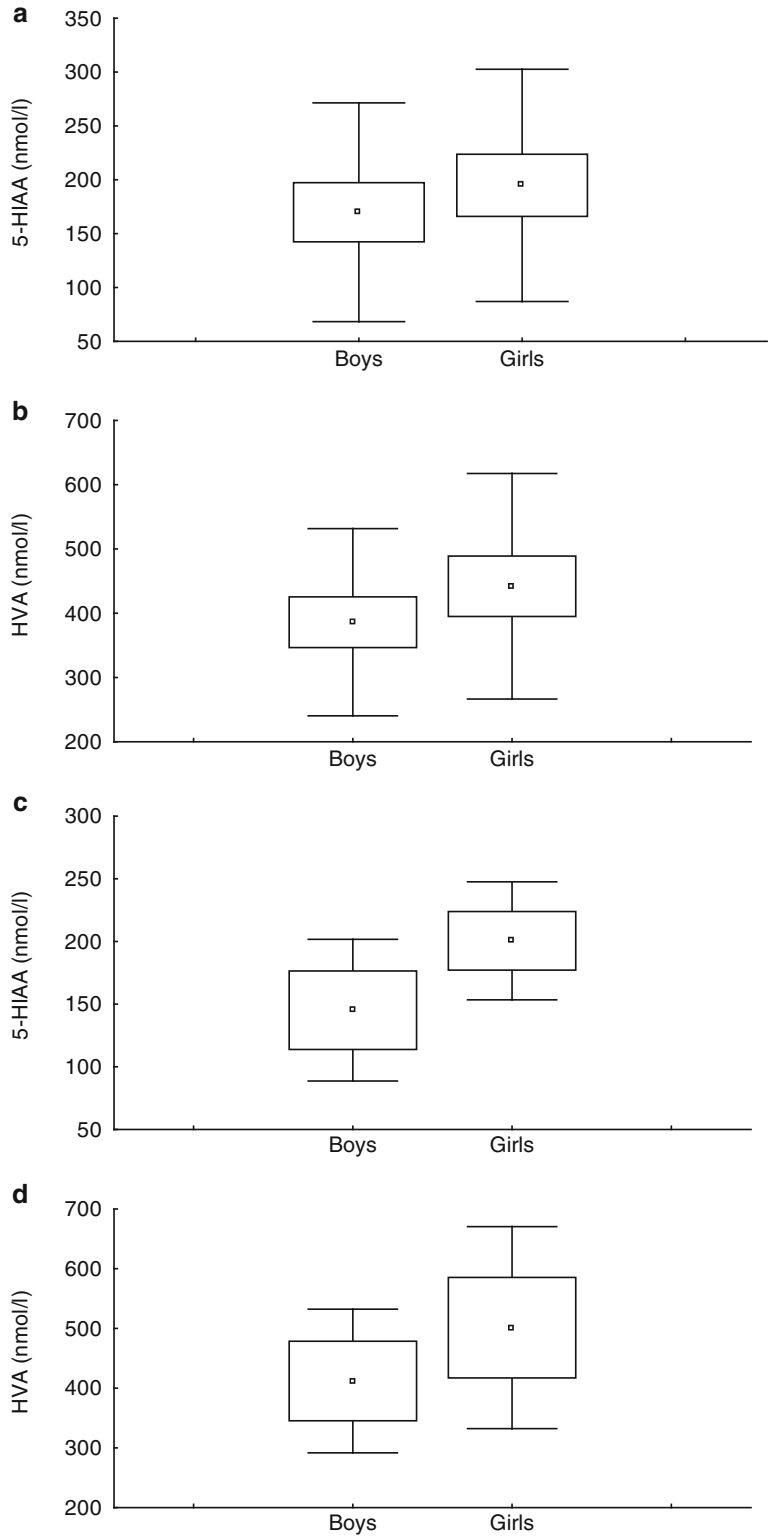

Fig. 3 Gender differences in homovanillic acid (HVA) and 5-hydroxyindoloacetic acid (5-HIAA) concentrations in boys and girls; (**a**) – 5-HIAA concentration across all ages in the entire study group; (**b**) – HVA concentration across all ages in the entire study group; (**c**) – 5-HIAA concentration in the singled out group of 1–4 years of age; and (**d**) – HVA concentration in the single out group of 1–4 years of age

establishing the reference ranges of a neurometabolic biomarker in CSF. Nonetheless, CSF monoamine metabolite analysis has become routine in pediatric neurology (Pearl et al. 2006). Although these metabolites have been characterized in children, the knowledge on their variation by age and sex is limited and the reference ranges for age-subgroups, representing distinct life periods, vary widely.

The present study revealed a number of age and gender-related differences in the CSF concentration of monoamine metabolites. Of the two examined monoamines, serotonin was most affected by age and sex. According to other authors, both dopamine and serotonin metabolites are the highest at birth and then decline across the sequential life periods (Kurian et al. 2011). The decline is expected to be the steepest in the first 12 months of life. We confirmed that for 5-HIAA, as we observed a significant age-related decrease in 5-HIAA across all three age groups. Our finding is consistent with the observations of other authors who have demonstrated the highest 5-HIAA levels in infancy and an age-related decline thereafter, up to early adulthood (Hyland 2006; Blau et al. 2005). There is ample evidence that serotonin is transiently synthesized at high levels in young children and this overactivity declines with time in healthy children (Dennis et al. 2013; Van Goozen and Fairchild 2006).

Opposite to 5-HIAA, HVA did not consistently decrease in the firsts two life periods examined, up to 4 years of age. We observed a postnatal reduction of HVA up to the age of 6 months, followed by a slight increase in the metabolite within further 6 months of life, up to the age of 12 months. Later on, HVA remained unaltered until the fourth year of life; thereafter gradually decreasing. According to other authors, a continuous decrease of HVA is expected in the first years of life (Hyland 2006; Kurian et al. 2011), which is in partial agreement with the present results. The discrepancy concerning the waning HVA decline beyond the first 6 months of life observed in the present study may stem from the number of children examined in both article quoted above, which was rather small. Of note, Hyland (2006) has also found that a gradual decrease of HVA is not as steep as that of 5-HIAA. The findings confirm the assumption that dopamine and serotonin, and their degradation products are differentially expressed during development (Whitaker-Azmitia 2005; Goldman-Rakic and Brown 1982). A clear reduction in serotoninergic neurons at the early age is not paralleled by a decrease in dopaminergic pathways which are involved in the control of crucial functions in the post-natal period such as movement and regulation of the endocrine, limbic and cardiovascular systems. The serotonergic system, on the other hand, affects the neuronal development, usually terminated early after birth, and emotions and behavior, rapidly changing during infancy (Dennis et al. 2013; Maciag et al. 2006). Serotonin plays a role of a neurotransmitter and neurogenic modulator of dendrite growth, interacting with brain monoamines (Dennis et al. 2013; Thomson and Stanwood 2009).

The present study demonstrates a significant correlation between HVA and 5-HIAA, largely confirming that both pathways are interdependent. Although the activity dynamics of both mediators varied in different age-groups, the HVA/5-HIAA ratio in the whole examined group was not age-related. The HVA/5-HIAA and age correlated only in children below 12 months of age. Later on, this ratio remained stable, as HVA and 5-HIAA decreased proportionally.

We have also analyzed gender-related differences in the concentration of both metabolites. Some authors argue that dopamine, in parallel with testosterone, is responsible for male sexual behavior (Dennis et al. 2013). We were unable to confirm that assumption either in the whole group examined or in the defined age-subgroups. The only significant gender-related difference was noted in the 5-HIAA concentration could be observed in children 1–4 years of age. The dopaminergic pathway was unrelated to gender, although there was an insignificant trend toward a lower HVA concentration in boys. Accordingly, Dennis et al. (2013) have shown that women have a lower

catecholamine concentration in the raphe nucleus and higher serotonin and 5-HIAA concentrations in the raphe nucleus and hypothalamus. Montoya et al. (2012) have attributed gender-related differences in the occurrence of aggressive behaviors to the disparities in serotonin formation, which, in turn, regulates testosterone and prolactin release in both sexes. Additionally, Biver et al. (1996) have shown that binding capacity of 5-HT receptors is higher in boys than girls (Biver et al. 1996). Aitkenhead and Heales (2013) have found a correlation between prolactin concentration and age and sex. Prolactin concentration drops in the neonatal period and remains then stable up to the fifth year of age; the changes going in parallel with dopamine release. Those observations are consistent with our present findings concerning the HVA concentrations in the respective age-groups. In adolescents aged 13–17, prolactin concentration is significantly higher in girls than boys (Aitkenhead and Heales 2013). We also observed a lower mean HVA concentration in boys, but the difference between girls and boys did not reach statistical significance. That could be attributed to the catechol-*O*-methyltransferase (COMT)-related dopamine metabolic pathway leading to the formation of norepinephrine and further epinephrine, being more active in boys than girls. The presence of sexual dimorphisms in the healthy human basal ganglia, notably regarding the dopaminergic system, which may result in differences in dopamine formation, has been confirmed by other authors. Apparently, females have a higher number of dopaminergic neurons in substantia nigr a than males (Smith and Dahodwala 2014).

5 Conclusions

The evidence presented herein supports the assumption that there are age and gender-related differences in the formation and metabolism of dopamine and serotonin in human brain. In this respect, the neural system is sexually dimorphic and probably interrelated with the gonadal steroid hormones. These effects have important behavioral consequences and implications for the potential gender differences in susceptibility to certain diseases. From the clinical perspective, however, quantitative differences in the concentration of dopamine and serotonin metabolites between the girls and boys were not great. The age-related correlations were much stronger and should be taken into consideration in clinical settings. Since biogenic amines in CFS fluctuate, there is a need to generate detailed reference values on the basis of large, diverse populations to allow for clinically accurate and physiologically relevant interpretation of patient results.

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

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The Influence of Primary Cytomegalovirus or Epstein-Barr Virus Infection on the Course of Idiopathic Thrombocytopenic Purpura

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Abstract

Idiopathic thrombocytopenic purpura (ITP) in children is usually triggered by a viral infections such as cytomegalovirus (CMV) or Epstein-Barr virus (EBV) infection. The aim of this study was to assess the frequency of CMV and EBV infections in children with first relapse of ITP, and the influence of these infections on the course and response to treatment of ITP. Sixty patients (30 boys and 30 girls) with ITP were enrolled into the study. We found that the age at the onset of ITP was from 1 month to 17 years (mean 7.0 $\pm$ 5.7 years), the platelet number was from 1 to 79 $\times$ 10^9/L (mean 18.1 $\pm$ 19.0 $\times$ 10^9/L) at the time of diagnosis and it increased from 17 to 395 $\times$ 10^9/L (mean 134.4 $\pm$ 81.2 $\times$ 10^9/L) ($p < 0.05$) after the first course of therapy. Forty seven patients required pharmacological treatment, the duration of the treatment was from 2 to 25 days (mean 6.1 $\pm$ 4.1 days). Relapses were observed in 27 (45 %) of the patients. Active CMV infection was found in 19 patients (31.7 %), EBV infection in 5 patients (8.3 %), and both infections concomitantly in 1 patient (1.7 %). The group of patients with CMV or EBV infection ($n = 25$) did not differ from the patients free of infection ($n = 35$) in regard to the age, number of platelets at onset, duration of treatment, number of platelets after treatment, number of relapses, and the interval between the onset and first relapse. In conclusion, active CMV or EBV infection is common in children with ITP. These infections do not seem to have an appreciable bearing on the clinical course and the response to treatment on children with ITP.

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Keywords
Children • Cytomegalovirus • Ebstein-Barr virus • Idiopathic thrombocytopenic purpura • Infection

1 Introduction

Idiopathic thrombocytopenic purpura (ITP) is the most common platelet bleeding disorder characterized by an isolated decrease of platelet count below 100×10^9/L (Rodeghiero et al. 2009). The incidence of ITP in the pediatric population is 4/100,000 per year. The peak age of onset is between 2 and 6 years of age. The symptoms of hemorrhagic disorder usually occur when platelet count falls below 30×10^9/L (Adamowicz-Salach et al. 2008). It is believed that ITP occurs when antibodies directed against platelet surface glycoproteins: GP IIb/IIIa, GP Ib/IX, and GPV are produced, and the plates are destroyed in the reticuloendothelial system (Rodeghiero et al. 2009). ITP is preceded by infection in 60–80 % cases. Steroids and intravenous immunoglobulins are used as the first line treatment of immune thrombocytopenia, while immunosuppressive drugs and monoclonal antibodies are applied in refractory cases and splenectomy in chronic ITP (Rodeghiero 2008; Tarantino and Goldsmith 1998; Shad et al. 2005; Blanchette et al. 1994). Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) are DNA viruses belonging to the Herpesviridae family. The EBV virus, called human herpesvirus type 4, belongs to Gammaherpesviridae, while the CMV virus, called human herpesvirus type 5, belongs to the Betaherpesviridae family. These viruses, replicating in leukocytes, may have an omnidirectional influence on immunological processes (Banks and Rouse 1992; Rice et al. 1984). The primary viral infection is often asymptomatic. Thereafter, however, viral latency in macrophages, T lymphocytes CD8+, and endocrine cells may occur. In terms of serological status, infections caused by viruses mentioned above are divided into primary, reactivated, persistent, history, or just susceptibility to infection (seronegative). It is believed that CMV and EBV viruses may be a common cause of autoimmune thrombocytopenia due to the phenomenon of antigenic mimicry stimulating the production of antiplatelet antibodies (Sheng Yu et al. 2008; Likic and Kuzmanic 2004, Wu et al. 2013; Cines et al. 2009). The destructive role of T cells and impaired production of platelets are mentioned as the processes responsible for the occurrence of ITP (Rodeghiero et al. 2009).

The aim of the present study was to determine the prevalence of CMV and EBV primary infections in children with the onset of immune thrombocytopenia and to assess the influence of these infections on the clinical course and response of the thrombocytopenia to treatment.

2 Methods

The study was approved by the Ethics Committee of the Medical University of Warsaw in Poland. Sixty patients were enrolled into the study: 30 boys and 30 girls hospitalized with the diagnosis of ITP in the Department of Pediatrics, Hematology and Oncology, Medical University of Warsaw, Poland, from March 2008 to December 2009. The following parameters were analyzed in all patients: presence of CMV or EBV infection, age at the onset of disease, platelet count at the time of diagnosis, duration of treatment, medications used (prednisone, methylprednisolone pulses, or intravenous immunoglobulin), response to treatment (assessed as the number of platelets after treatment), number of relapses, and time to the first relapse. Specific antibodies against viral antigens were determined with immmunofluorescence assays (Abbott Diagnostics; Sligo, Ireland) in serum samples collected from the patients at the time

of diagnosis before initiation of the specific therapy. Viral nucleic acids in the blood were detected by polymerase chain reaction (PCR) (LightCycler 2; Gene Proof, Brno, Czech Republic). The presence of CMV DNA was detected using CMV DNA Quantitation (QT) real-time PCR and that of the EBV DNA by nested PCR. Active primary cytomegalovirus infection was diagnosed when CMV DNA was identified or the level of IgM antibodies was greater than 0.5 of the avidity index. Active primary EBV infection was diagnosed in the presence of EBV DNA in the PCR test or viral capsid antigens (VCA) IgM and IgG greater than 1.1 standard deviation.

The results were presented as means ± SD. Normality of distribution was analyzed using the Shapiro-Wilk or Kolmogorov-Smirnov test. Normally distributed data were compared with a *t*-test for independent groups, and other than normally distributed data using the Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

3 Results

The age at onset of ITP ranged from 1 month to 17 years, mean of 7.0 ± 5.7 years, the platelet count at the moment of diagnosis was from 1 to 79 × 10^9/L, mean 18.0 ± 19.0 × 10^9/L and it increased from 17 to 395 × 10^9/L (mean 134.4 ± 81.2 × 10^9/L) ($p < 0.05$) after the first course of therapy. Forty seven out of the 60 examined children (78.3 %) required pharmacotherapy. The mean treatment duration was mean 6.1 ± 4.1 days. Relapses were observed in 27 of 60 (45 %) patients. Active infection with CMV or EBV viruses was observed in 25 of the 60 (41.7 %) patients. Nineteen (31.7 %) patients had active CMV infection, 5 patients (8.3 %) had EBV infection, and 1 (1.7 %) child was infected with both viruses. Of the 20 patients infected with CMV, the presence of CMV DNA was found in 11 children, IgM antibodies were found in 3 children, and in 6 children were positive for both IgM and CMV DNA. Of the 6 patients with EBV infection, 5 children had IgM antibodies and EBV DNA in blood, and 1 child had only IgM antibodies. Diagnostic criteria of CMV and EBV infection in the analyzed group are shown in Table 1. Table 2 presents the data of patients with ITP with and without EBV/CMV infection. The patients with CMV and/or EBV infection did not differ from patients without these infections in terms of age, platelet count at the time of diagnosis, and duration of treatment. Patients with CMV and/or EBV infection had a lower platelet count after the treatment, a higher number of relapses, and a shorter interval between the first and second projection

Table 1 Diagnostics of CMV and EBV infection in children with ITP

	PCR	Antibodies	PCR and antibodies	Overall
CMV	11 (55 %)	3 (15 %)	6 (30 %)	20 (100 %)
EBV	0 (0 %)	1 (17 %)	5 (83 %)	6 (100 %)

CMV cytomegalovirus, *EBV* Epstein-Barr virus, *ITP* idiopathic thrombocytopenic purpura, *PCR* polymerase chain reaction

Table 2 Clinical data of ITP patients with and without CMV/EBV infection

	Age (yr)	Platelet count at diagnosis (×10^9/L)	Treatment duration (days)	Platelet count after treatment (×10^9/L)	Number of relapses	Interval from onset to first relapse (months)
With CMV/EBV	7.0 ± 6.8	18.8 ± 21.5	6.6 ± 1.7	123.6 ± 68.8	2.9 ± 1.6	1.1 ± 1.3
Without CMV/EBV	7.0 ± 4.9	17.5 ± 17.4	6.8 ± 5.3	142.1 ± 90.3	2.0 ± 2.3	1.7 ± 2.3

No statistically significant differences were noted in any of the parameters above outline between the patients with and without; CMV/EBV infections. *ITP* idiopathic thrombocytopenic purpura, *CMV* cytomegalovirus, *EBV* Epstein-Barr virus

Table 3 Symptoms of infectious mononucleosis in patients treated for ITP with diagnosed CMV/EBV infection

	Hepatosplenomegaly	Lymphadenopathy	Elevated liver enzymes	Hyperleukocytosis
CMV	0	1	1	0
EBV	1	2	2	0

ITP idiopathic thrombocytopenic purpura, *CMV* cytomegalovirus, *EBV* Epstein-Barr virus

Table 4 Treatment of the first relapse of ITP

	Methylprednisolone or prednisone		Intravenous immunoglobulins		Without treatment
	Patients (n)	Treatment duration (days)	Patients (n)	Treatment duration (days)	Patients (n)
With CMV/EBV	12	5.8 ± 1.6	9	4.3 ± 1.4	6
Without CMV/EBV	19	8.1 ± 5.6	7	3.3 ± 1.3	9

No statistically significant differences were noted in any of the parameters above outline between the patients with and without CMV/EBV infections. *ITP* idiopathic thrombocytopenic purpura, *CMV* cytomegalovirus, *EBV* Epstein-Barr virus

compared with those without these infections, although the differences were not statistically significant. Clinical symptoms of infectious mononucleosis in patients with ITP and concomitant CMV or EBV infections are presented in Table 3. Only were a few patients symptomatic. Hepatosplenomegaly occurred in 1 patient, severe lymphadenopathy in 3 patients (1 with CMV infection and 2 with EBV infection), elevated liver enzymes in 3 patients (1 with CMV infection and 2 with EBV infection), none of the patients had hyperleukocytosis. Hypertransaminasemia in these patients was mild. These symptoms occurred more frequently in patients infected with EBV than CMV (5 *vs.* 2, respectively).

Table 4 classifies patients with autoimmune thrombocytopenia according to applied therapy. Thirty one (51.7 %) patients received treatment with glucocorticosteroids and 16 (26.7 %) were treated with immunoglobulins. Although the patients treated with glucocorticosteroids, both with CMV/EBV infection and without these infections, required longer treatment than those treated with immunoglobulins, the differences between the time-course of both methods did not reach statistical significance. Among the 31 patients treated with glucocorticosteroids, patients without viral infections required shorter treatment, and among the patients treated with immunoglobulins, those with viral infections required longer therapy, although in both cases the differences did not reach statistical significance.

Bleeding complications occurred in 6 patients (10 %): 1 patient experienced a life-threatening bleeding from the upper gastrointestinal tract, 1 patient had hematuria, 2 massive epistaxis, and 2 girls massive bleeding from the uterus during menstruation. Among the patients with bleeding complications, the primary EBV infection was present in the patient with gastrointestinal bleeding, the patient with hematuria, and in one of the girls with vaginal bleeding. One patient with bleeding from the nose had primary CMV infection. Four of the six (66.7 %) patients with bleeding complications in our study group had an active primary infection with CMV or EBV viruses.

4 Discussion

In the present study, we assessed the incidence of primary CMV and EBV infections in patients with ITP and the influence of CMV/EBV infection on the clinical course and response to treatment. CMV and EBV viruses are often cited as a significant causes of ITP in children (Blanchette and Carcao 2003; Watts 2004; Rand and Wright 1998; Sheng Yu et al. 2008). Mitura-Lesiak et al. (2004) have found CMV infection in 19 of 107 (26.4 %) and EBV in 9 of 107 (9.7 %) children with ITP. The percentage of CMV and EBV infections in patients of that study is comparable with the results obtained in the present study.

None of our patients were diagnosed with clinically overt infectious mononucleosis prior to ITP. Viral infection was identified solely on the basis of laboratory tests, only had individual patients clinical symptoms that could be associated with EBV/CMV infection (lymphadenopathy, hepatosplenomegaly, hypertransaminasemia, or hyperleukocytosis). These symptoms occurred more frequently in patients infected with EBV. In the studies by other authors, thrombocytopenia has been followed by clinically overt infectious mononucleosis (Likic and Kuzmanic 2004; Takahashi et al. 2008). In the literature, CMV and EBV infections are described as risk factors for resistance to treatment, severe course, hemorrhagic complications, and death in children and adults with ITP (DiMaggio et al. 2009; Brodkiewicz et al. 2009; Walter et al. 2004). Miyahara et al. (1997) have described a case of severe thrombocytopenia with myelodysplasia in an adult patient with CMV infection. In the present study, patients infected with these viruses did not require longer treatment, but the response to the treatment was worse and relapse occurred earlier. We demonstrate the efficacy of both glucocorticosteroids and immunoglobulins in the treatment of ITP caused by CMV and EBV infections in comport with other authors (Blanchette et al. 1994; Tavil et al. 2008; Takahashi et al. 2008; Wu et al. 2013). In the investigated group the children with EBV/CMV infections had a higher number of relapses than those without infections (2.9 ± 1.6 *vs.* 2.0 ± 2.3, respectively) and a shorter interval between onset and first relapse, although the differences were insignificant. It is possible that the lack of statistical significance reflects a limited number of the subjects enrolled. In our group, the response to both treatment options used was good. A similar response to therapy in patients infected with CMV and EBV, and without these infections may result from the occurrence of a number of cases resistant to treatment without EBV and CMV infections. DiMaggio et al. (2009) have described a group of four patients with ITP associated with CMV infection, treated with the antiviral drug ganciclovir to achieve remission; glucocorticosteroids impaired the outcome of therapy. In the present study, none of the patients required antiviral therapy and the standard treatment for ITP was effective. We found that CMV or EBV infection is a risk factor for bleeding complications. Brodkiewicz et al. (2009) have described a case of fatal bleeding into the central nervous system in a 5-year-old girl with ITP co-infected with EBV. In the literature, deaths caused by ITP are rare, accordingly we did not note any fatal clinical course.

In conclusion, active primary CMV or EBV infection is common in children with ITP. These infections influence the clinical course and the response to treatment in children with ITP. CMV or EBV infection seems a risk factor for hemorrhagic complications.

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

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