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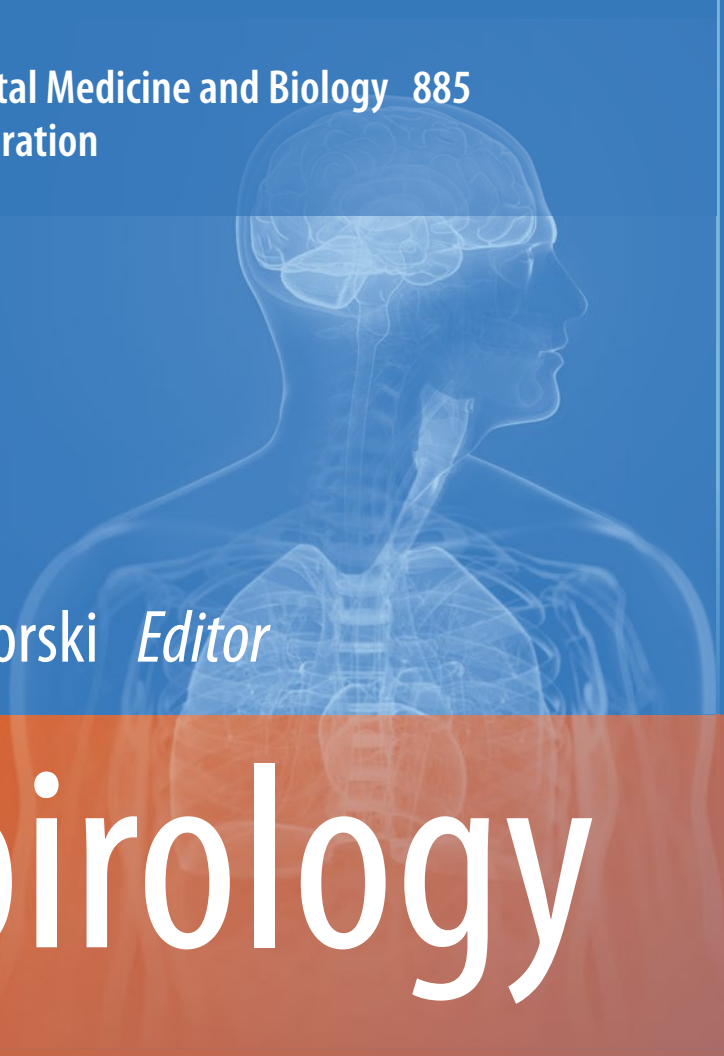

Mieczyslaw Pokorski *Editor*

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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

Contents

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Growth, Nutritional Status, and Pulmonary Function in Children with Chronic Recurrent Bronchitis

Wioleta Umławska and Anna Lipowicz

Abstract

Bronchitis is a common health problem in children. Frequent bronchitis in infancy increases the risk of developing chronic respiratory diseases. The aim of the study was to assess the level of growth and the nutritional status in children and youths with special regard to the level of body fatness assessed by measuring skin-fold thickness. Relationships between somatic development, pulmonary function and the course of the disease were also explored. The study was carried out using anthropometric and spirometric measurements and also information on the severity and course of the disease in 141 children with chronic or recurrent bronchitis. All of the subjects were patients of the Pulmonary Medicine and Allergology Center in Karpacz, Poland. The mean body height did not differ significantly between the children examined and their healthy peers. However, the infection-prone children had excessive body fatness and muscle mass deficiency. The increased level of subcutaneous adipose tissue occurred especially in children with short duration of the disease, i.e. a maximum of 1 year. The functional lung parameters were generally normal. The presence of atopic diseases such as allergic rhinitis or atopic dermatitis did not impair the course of the children's somatic development. Also, long-term disease or the presence of additional allergic diseases did not impair lung function in the examined children. Taking appropriate preventive measures is recommended to achieve and maintain normal body weight in children who receive therapy due to bronchitis.

Keywords

Anthropometry • Body composition • Bronchitis • Children • Fatness • Somatic growth • Spirometry

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1 Introduction

Recurrent bronchitis is a common health problem in children. Studies indicate that approximately 30 % of children in the first year of life suffer from at least once episode of obstructive bronchitis (Martricardi et al. 2008; Wright 2002). Obstructive bronchitis leads to long-term changes in the bronchial tree, including bronchial hyper-reactivity and a tendency of the bronchi to obstruct during subsequent infections. Relapses of the disease are reported on average in 30–90 % of children, especially in the first years of life. In subsequent years, the number of bronchial obstruction incidents usually decreases and amounts to 30 % on average in school-age children (Krych et al. 2005).

Frequent bronchitis in infancy increases the risk of developing chronic respiratory diseases (Illi et al. 2006; Horak et al. 2003). Children who in the first years of life suffer from recurrent or chronic bronchitis accompanied by wheezing have an increased risk of bronchial asthma (Stern et al. 2008). The risk of bronchial asthma is increased by simultaneous atopy (Horak et al. 2003).

The results of population studies prove a clear link between body weight and the level of fatness and the incidence of chronic respiratory diseases (Jędrychowski et al. 1998; Must 1996). Studies conducted in a group of more than 70,000 people within the Italian National Health Survey demonstrated a U-shaped type of relationship between body weight and the incidence of chronic respiratory disorders; the disorders occur more frequently in both underweight and overweight subjects (Negri et al. 1988). This relationship has also been confirmed by previous observations of the nutritional status of patients with bronchitis (Viola et al. 2008; Somerville et al. 1984). Currently, however, most studies, both cross-sectional and longitudinal, show that excessive body weight increases the risk of bronchial asthma, wheezing and chronic lower respiratory tract infections (von Mutius et al. 2001).

The assessment of the nutritional status and the level of growth in children with chronic bronchitis, in contrast to bronchial asthma, is the subject of relatively few auxological studies. Previous studies on the somatic development of children with bronchitis have mostly been performed among preschool-aged children, and the results are inconclusive. Some studies indicate the lack of disorders, while others point to growth deficiencies (Pawlińska-Chmara and Wronka 2007; Ford et al. 2001; Dumas et al. 1997).

Literature data also suggest that atopy *per se*, often coexisting in the course of recurrent respiratory infections, may temporarily or permanently disrupt the somatic development (Sant'Anna et al. 1996). In children with allergic diseases such as allergic rhinitis or atopic dermatitis, the incidence of short stature is from 3 to even 5 times higher than that observed in the general population. However, the reason for this phenomenon remains unclear (Baum et al. 2002).

In light of these often conflicting studies, it was considered appropriate to investigate a group of children diagnosed and treated for chronic or recurrent bronchitis. The aim of the study was to assess the level of growth and the nutritional status in children and youths, with special regard to the level of body fatness assessed by measuring skin-fold thicknesses. Relationships between somatic development, pulmonary function and the course of the disease were also explored.

2 Methods

The study protocol was approved by the Institute of Mother and Child Ethics Committee, and written informed consent was obtained from the parents of each subject.

The study was carried out using anthropometric measurements and information on the severity and course of the disease in 141 children with chronic or recurrent bronchitis. The sample comprised 77 boys and 64 girls, from 6 to 18 years of age (Table 1). All of the subjects were patients of the Pulmonary Medicine and Allergology Center in Karpacz, Poland. The study lasted from May 2005 to October 2005.

Table 1 Demographic data of the children examined

	n	Mean age ± SD *(years)*	Age range *(years)*
Boys	77	11.8 ± 3.1	6.5–18.0
Girls	64	11.9 ± 3.0	6.5–17.5
Total	141	11.8 ± 3.2	6.5–18.0

The mean age at which the first symptoms of bronchitis were reported in the children examined was 5.3 ± 3.8 years, and ranged from 0.5 to 11 years. The mean duration of the disease was 6.5 ± 3.9 years, and ranged from 6 months to 12 years. It was found that recurrent bronchitis with chronic coughing, sputum production, and breathlessness occurred at least 4 times a year.

The subjects were assigned to three categories in terms of the duration of the disease: (1) less than 1 year (mean 0.9 ± 0.4 years); (2) from 1 to 5 years (mean 3.3 ± 1.1 years); and more than 5 years (mean 9.1 ± 2.7 years). This was done to distinguish the children who had been suffering from bronchitis for a longer time, over 5 years, and those who had been diagnosed relatively recently, within a year before the start of the present study. Of the subjects included in the study 9.9 % had been suffering from bronchitis for less than 1 year, 30.5 % for between 1 and 5 years, and 59.6 % for more than 5 years.

Fifty of the children included (35.5 %) demonstrated allergic diseases such as allergic rhinitis and atopic dermatitis. Seventy nine children (56.0 %) had a positive family history for atopic diseases. There were no apparent cases of bronchial asthma.

Based on the information from the medical history taken from the children's parents and on the analysis of medical records, it was found that the treatment of children included antibiotics, mucolytics, and anti-inflammatory medications and bronchodilators. Sixty four children (45.4 %) were treated with inhaled corticosteroids, 51 with a dose of 200 mg per day and 13 subjects with a dose of 200–400 mg per day. Inhalation therapy was carried out for 4 years on average (min. 0.5 year, max. 5 years).

The subjects also suffering from allergic rhinitis and atopic dermatitis used nasal sprays in their treatment. The preparations are synthetic corticosteroids with topical anti-inflammatory and anti-allergic action, as well as ointments with topical steroidal anti-inflammatory, antipruritic, and immunosuppressive action.

Anthropometric parameters were measured by trained anthropologist in accordance with the procedures described by Martin and Knusmann (1988). The following data were recorded: height, weight, sagittal chest depth, transverse chest width, triceps skin-fold thickness, subscapular skin-fold thickness, abdominal skin-fold thickness, the sum of the three skin-fold thicknesses, and upper arm circumference. Height was measured to the accuracy of 1 cm. Weight was measured to an accuracy of 0.1 kg. Widths were measured with large bow callipers to an accuracy of 1 mm. Skin-fold thicknesses were measured with an instrument produced by Holtain (Crymych, Pembs, UK) to the accuracy of 0.2 mm.

Body mass index (BMI; kg/m^2) was used as an indicator of relative weight. Children were classified as undernourished if they had a BMI less than the 5th percentile, properly nourished if they had a BMI between the 5th and 85th percentiles, and overweight if they had a BMI greater than the 85th percentile. Upper arm muscle circumference (AMC) was calculated using the following formula: $AMC = AC - (\pi \times TST)$; where AC represents arm circumference and TST represents triceps skinfold thickness. Body composition was estimated using the upper arm muscle area and upper arm fat tissue area. Upper arm muscle area (UAMA) was calculated using the following formula: $UAMA = [AC - (\pi \times TST)]^2$. Upper arm fat tissue area (UAFTA) was calculated using the following formula: $UAFTA = UAA - UAMA$; where UAA represents upper arm area. Anthropometric parameters were expressed in terms of standard deviations away from the age- and sex-specific mean reference values established for the Polish population by Palczewska and

Niedźwiedzka (2001); being widely recognized as valid for research purposes.

Spirometric measurements were performed. Data recorded included forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), maximum midexpiratory flow over the middle half of the FVC, ($FEF_{25–75}$), and forced respiratory volume in 1 s % of vital capacity (FEV_1%VC). All spirometric parameters were measured using a Jaeger spirometer (Toennis, Hochberg, Germany) in accordance with the procedures recommended by the Polish Phthysiopneumological Society. All results were recorded as percentages of the predicted values, standardized for age, gender, and height.

Data were given as means ± SD. Distribution of values for somatic traits was evaluated using the Kołmogorov-Smirnov test. Differences between the values obtained for the children examined and the reference values for the Polish population as established by Palczewska and Niedźwiedzka (2001) and between boys and girls were evaluated using a *t*-test or, for asymmetrical distributions, the Mann–Whitney test. Differences between boys and girls in the groups in terms of nutritional status were compared using the χ^2 test. The effect of selected factors associated with the course of the disease on somatic and spirometric parameters, taken as dependent variables, was determined using multivariate regression analyses. The independent variables included the duration of the disease and atopy. Standardized beta coefficients were used to facilitate comparison of the strength of the correlation of individual independent variable with the dependent variables recorded in different units. Differences were considered significant at $p < 0.05$. All analyses were carried out using an Statistica 9.0 software package.

3 Results

The results of somatic and functional measurements are shown in Tables 2 and 3. The mean body height in subjects suffering from bronchitis did not differ significantly from that in the reference group. Severe growth disorders and short stature, however, were found in seven of the examined children (5 %) (Figs. 1 and 2).

Compared with the reference group of healthy children, the children with chronic recurrent bronchitis demonstrated a disturbed chest structure. The sagittal chest depth was increased in relation to the transverse chest width, making the chest index higher than that present in the

Table 2 Anthropometric parameters of 141 children and adolescents with chronic recurrent bronchitis, expressed in terms of standard deviations away from the mean of reference values for the population of Poland

Parameter	Mean Z score ± SD	Range	p[a]
Body height	0.08 ± 1.17	−2.42–3.45	0.559
Body weight	0.25 ± 1.17	−2.14–3.52	**0.013**
Body mass index	0.28 ± 1.18	−2.07–3.35	**0.005**
Transverse chest width	−0.03 ± 1.19	−3.52–3.25	0.791
Sagittal chest depth	0.15 ± 1.32	−2.37–4.97	0.153
Chest index	0.22 ± 1.25	−2.66–5.23	**0.038**
Triceps skinfold thickness	1.39 ± 1.75	−1.30–5.12	<**0.001**
Subscapular skinfold thickness	0.87 ± 1.54	−1.22–6.09	<**0.001**
Abdominal skinfold thickness	1.65 ± 2.19	−1.34–7.97	<**0.001**
Sum of three skinfold thicknesses	1.29 ± 1.56	−0.83–6.88	<**0.001**
Upper arm circumference	0.21 ± 1.20	−2.11–3.43	**0.035**
Upper arm muscle circumference	−0.65 ± 1.11	−3.27–2.51	<**0.001**
Upper arm fat tissue area	1.06 ± 1.58	−1.10–5.47	<**0.001**
Upper arm muscle area	−0.58 ± 1.05	−2.95–2.93	<**0.001**

Values are expressed as mean Z-score ± SD and range

[a]Student's *t*-test: mean Z-score = 0

Table 3 Spirometric indices, standardized for age and sex and expressed as % of predicted and range, examined in children with chronic recurrent bronchitis

	Boys	Girls	p-value
FVC	94.5 ± 13.9 (67–130)	93.5 ± 12.1 (65–122)	0.622[a]
FEV_1	96.3 ± 12.8 (66–129)	98.2 ± 3.9 (68–137)	0.405[a]
$FEF_{25–75}$	97.3 ± 22.6 (47–145)	100.7 ± 23.2 (55–164)	0.422[a]
FEV_1%VC	100.9 ± 11.0 (41–119)	103.4 ± 7.2 (88–128)	0.202[b]

FVC forced vital capacity, FEV_1 forced expiratory volume in 1 s, $FEF_{25–75}$ maximum midexpiratory flow over the middle half of the FVC, FEV_1%*VC* forced expiratory volume in 1 s % of vital capacity
[a]Student's *t*-test
[b]Mann-Whitney test

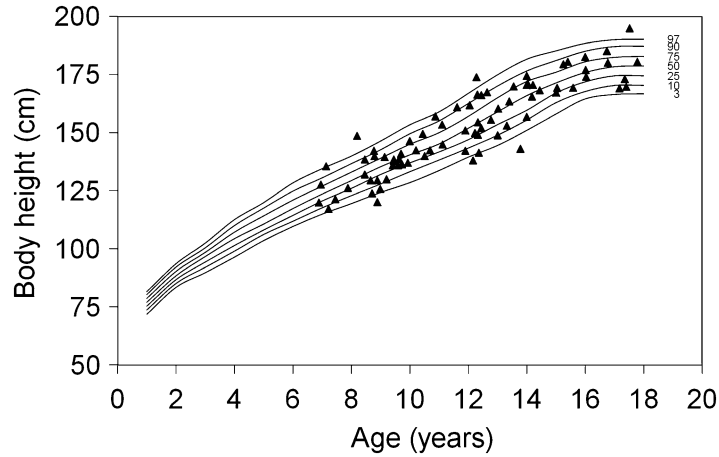

Fig. 1 Percentile chart of body height for boys with chronic recurrent bronchitis

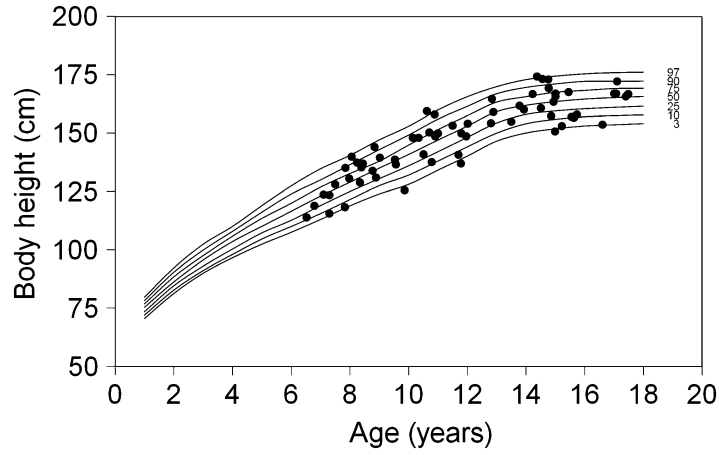

Fig. 2 Percentile chart of body height fro girls with chronic or recurrent bronchitis

reference group. The children with bronchitis had a stouter body appearance, as evidenced by higher values of body weight and arm circumference. The BMI was significantly greater, as also was adiposity measured by the upper arm, subscapular, and abdominal skin-fold thicknesses. Lean muscle mass, on the other hand, was significantly lower in the children with bronchitis than that in the reference group (Table 2).

Table 4 Relative body weight in children with recurrent bronchitis

	Boys	Girls	Total
Nutritional status	n (%)	n (%)	n (%)
Underweight (BMI below the 5th percentile)	1 (1.3)	2 (3.1)	3 (2.1)
Normal weight (BMI between the 5th and 85th percentiles)	56 (72.7)	47 (73.4)	103 (73.1)
Overweight (BMI between the 85th and 95th percentiles)	5 (6.5)	6 (9.4)	11 (7.8)
Obese (BMI over the 95th percentile)	15 (19.5)	9 (14.1)	24 (17.0)

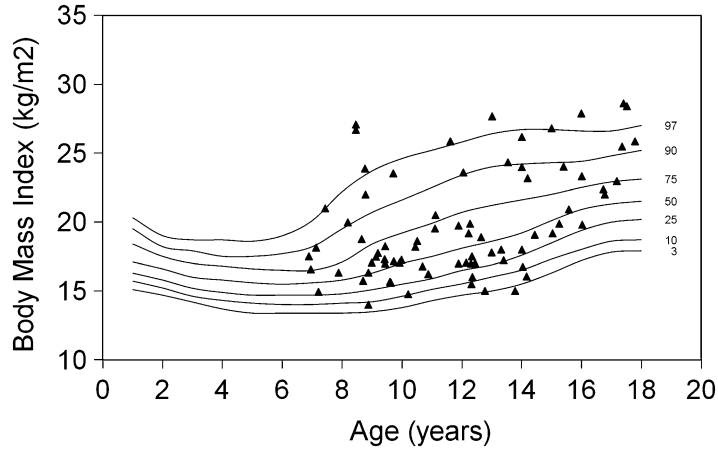

Fig. 3 Percentile chart of body mass index for boys with chronic or recurrent bronchitis

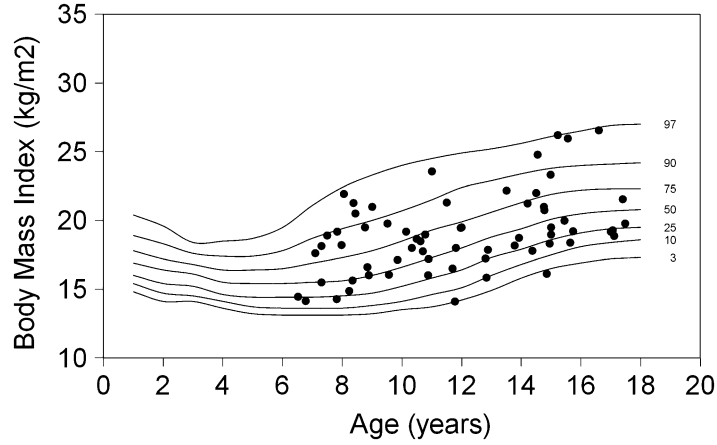

Fig. 4 Percentile chart of body mass index for girls with chronic or recurrent bronchitis

Percentile values of BMI show that there was a high prevalence of overweight and obesity (almost 25 %) and a relatively low proportion of underweight children (2 %) in the children with bronchitis (Table 4, Figs. 3 and 4). There were no significant inter-gender differences in the somatic traits or nutritional state in the children with bronchitis.

A relationship was observed between the chronicity of bronchitis, expressed as disease duration, and its co-occurrence with atopy, such as allergic rhinitis and atopic dermatitis, and with BMI and the level of fatness in the affected children (Table 5). The longer the disease duration, the lower were BMI, skin-fold thickness on the abdomen, and the sum of the three skin-fold

Table 5 Two-factor regression analysis of the relation of disease duration and atopy with indices of physical development and spirometry

	Duration of disease[a]		Presence of atopy[b]	
	Beta	p	Beta	p
Body height	−0.052	0.539	0.134	0.115
Body weight	−0.152	0.073	0.122	0.148
Body mass index	−0.173	**0.042**	0.085	0.317
Transverse chest width	−0.027	0.755	0.100	0.241
Sagittal chest depth	−0.131	0.122	0.109	0.199
Chest index	−0.141	0.098	0.074	0.381
Triceps skinfold thickness	−0.133	0.117	0.020	0.812
Subscapular skinfold thickness	−0.166	0.052	0.047	0.581
Abdominal skinfold thickness	−0.216	**0.050**	−0.024	0.828
Sum of three skinfold thicknesses	−0.177	**0.040**	0.027	0.753
Upper arm circumference	−0.138	0.105	0.052	0.543
Upper arm muscle circumference	−0.023	0.789	0.060	0.487
Upper arm muscle area	−0.060	0.481	0.049	0.570
Upper arm fat tissue area	−0.054	0.114	0.054	0.523
FVC%	0.081	0.343	−0.029	0.733
FEV_1%	0.051	0.551	−0.044	0.610
$(FEF_{25–75})$%	0.011	0.902	−0.044	0.604
FEV_1%VC	−0.033	0.702	−0.015	0.889

FVC forced vital capacity, *FEV1* forced expiratory volume in 1 s, $FEF_{25–75}$ maximum midexpiratory flow over the middle half of the FVC, *FEV1%VC* forced expiratory volume in 1 s % of vital capacity
Independent variables: [a]disease duration: 1 year, 1–5 years, and above 5 years
[b]Bronchitis without atopy and bronchitis with atopic conditions

thicknesses and *vice versa*. Thus, newly diagnosed children having a short duration of the disease had higher values of BMI and fatness. There was no relationship between the presence of additional atopic diseases and the level of somatic development of children.

Spirometric indices in the children with chronic recurrent bronchitis did not indicate any impairment of pulmonary function (Table 3). Nor was there any influence on spirometric indices of disease duration or the presence of additional allergic diseases (Table 5).

4 Discussion

Children suffering from chronic recurrent bronchitis did not deviate in terms of body height from healthy children. The percentage of children with short stature, which was 4 %, turned out to be similar to that observed in the general population (Palczewska and Niedźwiedzka 2001). That does not exclude the possibility that physical development and health in children with chronic bronchitis could be impaired, compared with the healthy peers, a few years on from the current sickness even if they recover from it. In particular, chronic bronchitis might increase susceptibility to bronchial asthma, atopy, and other allergic conditions in future years. This issue remains to be explored in alternative study designs. Pediatricians emphasize that the treatment of children up to 5 years of age with chronic recurrent bronchitis is problematic. At this age, diagnostic procedures are difficult and the diagnosis of bronchial asthma is usually made based on a thorough medical history. It is difficult to predict how many children would go on to develop asthma or in how many the symptoms would abate (Wright 2002).

The children with bronchitis we examined had a disharmony in the chest structure. Chest depth was increased in relation to width, resulting in a chest index higher than that in healthy peers. Such chest structure changes are also seen in

individuals with chronic respiratory disorders like cystic fibrosis or bronchial asthma (Umławska and Susanne 2008; Lopes et al. 2007). The most common abnormalities of the chest include: barrel or pigeon chest and Harrison's grooves (Gillam et al. 1970). These defects are caused by long-term exertion of respiratory muscles, bronchial obstruction, coughing attacks, and dyspnea (Lopes et al. 2007). Compared with healthy children, children with chronic bronchitis we examined had a stouter body silhouette, resulting from excessive body fatness. A quarter of the children were overweight or obese, while malnutrition was observed in only 2 % of them. They also had a deficiency of lean muscle mass.

Results of population studies indicate that people with excessive body weight are more susceptible to infectious diseases of the respiratory tract. A study on pre-pubertal children, which included more than one thousand subjects, has found that overweight and obese children suffer significantly more often from allergic diseases and respiratory infections, such as laryngitis, tonsillitis, tracheitis, and bronchitis, than underweight or normal weight children (Jędrychowski et al. 1998). The relationship between excessive body weight and respiratory diseases is not limited to the period of childhood. Greater body mass increases vulnerability to bronchitis and other infectious respiratory diseases also at later age. Preventive measures against the development of overweight and obesity in children and adolescents reduce the incidence of chronic respiratory diseases (Jędrychowski et al. 1998; Somerville et al. 1984).

Studies conducted in preschool children with chronic bronchitis also point to the presence of nutritional disorders, more often manifested as malnutrition rather than excessive body weight, due to the loss of both fat and muscle mass (Staszak-Kowalska 1993); which may be corrected by prolonged treatment. Malnutrition strongly correlates with deterioration of lung function (Viola et al. 2008).

The present study demonstrates a relationship between nutritional status and duration of the disease. The highest values of BMI and fatness concerned the children with the shortest, one-year duration of the disease. The result may point to the possible normalization of body weight in children under longer, regular medical care. A different interpretation is given for excessive body weight observed in a yearlong lasting asthma by Sadowska et al. (1986). The authors interpret the weight gain as a consequence of reduction in physical activity caused by increased parental concern about a sick child. A cross-sectional nature of the present study limits its interpretation in certain ways. It was not possible to evaluate growth and nutritional status at the time of bronchitis detection in a given child. We believe, however, that a large number of somatic measurements and their parsing analysis permit the accurate evaluation of physical development in relation to disease course.

5 Conclusions

Children with chronic recurrent bronchitis have no growth abnormality. They do have excessive body fatness and muscle mass deficiency. The increased level of subcutaneous adipose tissue occurs especially in children with a short, 1-year long duration of the disease. Spirometric indices are generally normal and do not worsen in the presence of allergic co-morbidities. The presence of atopic diseases such as allergic rhinitis or atopic dermatitis does not impair somatic development. Taking appropriate prophylactic measures is recommended to achieve and maintain normal body weight in children receiving treatment for bronchitis.

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

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Next-Generation Sequencing of 5′ Untranslated Region of Hepatitis C Virus in Search of Minor Viral Variant in a Patient Who Revealed New Genotype While on Antiviral Treatment

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Abstract

The role of mixed infections with different hepatitis C virus (HCV) genotypes in viral persistence, treatment effects, and tissue tropism is unclear. Next-generation sequencing (NGS), which is suitable for analysis of large, genetically diverse populations offers unparalleled advantages for the study of mixed infections. The aim of the study was to determine, using two different deep sequencing strategies (pyrosequencing – 454 Life Sciences/Roche and reversible terminator sequencing-by-synthesis by Illumina), the origin of a novel HCV genotype transiently detectable during antiviral therapy (pre-existing minor population *vs*. de novo superinfection). Secondly, we compared 5′ untranslated region (5′-UTR) variants obtained by the two NGS approaches. 5′ UTR amplification products from 9 samples collected from genotype 1b infected patient before, during, and after treatment (4 serum and 5 peripheral blood

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mononuclear cell – PBMC – samples) were subjected to the next-generation sequencing. The sequencing revealed the presence of two (454/Roche) and one (Illumina) genotype 4 variants in PBMC at Week 16. None of these variants were present either in the preceding or following samples as revealed by both platforms. 454/Roche sequencing detected 24 different 5'-UTR variants: 8 were present in serum and PBMC, 4 only in serum and 12 only in PBMC. Illumina sequencing detected 11 different 5'-UTR variants: 5 in serum and PBMC, 4 only in serum and 2 only in PBMC. Six variants were identical for both sequencing platforms. The difference in variants number was primarily due to variability in two 5'-UTR homopolymeric regions. In conclusion, longitudinal analysis of HCV variants, employing two independent deep sequencing methods, suggests that the transient presence of a different genotype strain in PBMC was a result of superinfection and not a selection of pre-existing minor variant.

Keywords
5' untranslated region • Hepatitis C virus • Next-generation sequencing • Mixed infection • Peripheral blood mononuclear cells

1 Introduction

Infection with mixed genotypes was reported to be present in 5–10 % of all hepatitis C virus (HCV)-positive patients (Bowden et al. 2005; Schroter et al. 2003). This form of infection is often accompanied by increased HCV RNA and aminotransferase activity and may result in the emergence of virulent recombinant strains (Blackard and Sherman 2007). The concomitant infection with two or more genotypes leads to direct competition since even minor differences in the replication rate of individual variants could result in the predominance of one strain over the other (Laskus et al. 2001; Laskus et al. 1996). However, some minor variants could persist in extrahepatic compartments such as lymphoid cells, which could provide specific replication benefits for these strains (Roque-Afonso et al. 2005).

Conventional method for HCV genotyping includes sequencing and analysis of conserved genomic regions by hybridization-based techniques (Nakatani et al. 2011). These techniques have limited sensitivity and may not detect minor variants. Higher sensitivity with respect to the minor strain detection may be provided by the next-generation sequencing (NGS); (Nakatani et al. 2011). High-throughput of NGS platforms generate millions of reads in a single sequencing run, facilitating in-depth sequencing of viral population (Liang et al. 2011). Thus, NGS can detect variants present at low frequencies, which would be undetected by the standard sequencing methods (Barzon et al. 2011; Simen et al. 2009).

The aim of this study was to determine, using two independent NGS strategies (pyrosequencing – 454 Life Sciences/Roche and reversible terminator sequencing-by-synthesis by Illumina), whether genotype 4 strain, transiently detectable in peripheral blood mononuclear cells (PBMC) in a patient infected with genotype 1b undergoing antiviral therapy, represented a pre-existing minor virus population, which was selected by treatment, or was a result of superinfection restricted to the PBMC compartment. Secondly, we compared 5'-untranslated region (5' UTR) variants obtained using two NGS approaches.

2 Methods

2.1 Subjects

The study was approved by the Internal Review Board at the Medical University of Warsaw, Poland (permit no. KB-0/23/09) and the patient provided informed consent. The subject was a 57-year-old male with chronic type C hepatitis who underwent 48-week antiviral therapy with pegylated interferon alpha-2a (Peg-Intron; Schering-Plough, Nenilworth, NJ), 150 μg per week and ribavirin (Rebetol, Schering Plough), 1200 mg daily, but did not achieve sustained virological response (SVR). Serum and PBMC samples were collected before (baseline), during (at Weeks 4, 8, 16, 24, and 48), and after completing the treatment (Weeks 60 and 72). The presence of HCV RNA was assessed by non-commercial PCR test (sensitivity: 50 copies/ml). The infecting genotype at baseline was 1b as assessed Versant HCV Genotype Assay (LiPA; Innogenetics, NV, Gent, Belgium) and the initial viral load was 2.2×10^6 copies/ml (Cobas Amplicor HCV Monitor Test v2.0; Roche Diagnostics, Pleasanton, CA). All HCV RNA positive samples were amplified and the genotype was determined by direct sequencing of the 5'-UTR. All samples contained the same genotype 1b, with a single exception of PBMC sample collected at Week 16, which contained genotype 4 variant.

2.2 5′-UTR Amplification and Next-Generation Sequencing

5'-UTR amplification was performed in all samples by RT PCR as described previously (Laskus et al. 2000). Samples which were positive for HCV RNA (baseline serum and PBMC, Week 4 serum and PBMC, Week 16 PBMC, Weeks 60 and 72 serum and PBMC) were analyzed by next-generation sequencing. For 454/Roche pyrosequencing, first round PCR products were subjected to 15 cycles of amplification with fusion primers with sequences of multiplex identifiers (MID) specific for each sample. The sequence-specific primers were as follows: forward primer 5'-ACTGTCTTCACGCAGAAAGCGTC-3' and reverse primer 5'-CAAGCACCCTATCAGGC-AGTACC-3'. Amplicon products were extracted from agarose gel by QIAquick Gel Extraction Kit (Qiagen; Hilden, Germany), purified by Agencourt AMPure XP PCR Purification system (Beckman-Coulter; Beverly, MA), and measured fluorimetrically using Quant-iT™ PicoGreen dsDNA Assay Kit (Molecular Probes; Eugene, OR) on QuantiFluor-ST Fluorometer (Promega; Madison, WI). 454/Roche pyrosequencing was performed following the amplicon processing protocol using GS Junior System (454/Roche; Branford, CT). To amplify and sequence 5'UTR region on Illumina platform, specific primers were designed (Fig. 1). Each primer contained: (1) sequences complementary to the adapters on a flow cell; (2) an 8 nt index sequence; (3) sequences corresponding to the Illumina sequencing primers; (4) 5'-UTR sequence specific primer. PCR was performed in 25 cycles, each comprised of three steps: 94 °C-1 min, 58 °C-1 min, and 72 °C- 1 min. Each 400 bp PCR product was extracted from agarose gel by QIAquick Gel Extraction Kit (Qiagen; Hilden, Germany) and quantified on Qubit 2.0 Fluorometer (Life Technologies-Invitrogen; Carlsbad, CA). The quality and average length of sequence library for each sample was assessed using a Bioanalyzer (Agilent Technologies; Santa Clara, CA). The indexed samples were pooled equimolarily and sequenced on Illumina MiSeq (301 base paired-end reads) according to the manufacturer's protocol (Illumina, San Diego, CA).

2.3 Data Filtering and Analysis

Sequencing reads that did not match the primer sequences or had undetermined bases (Ns) were excluded from further analysis "to diminish the contribution of false positive variants to genetic diversity" 1 % and 0.8 % cut-offs were applied, which corresponded to experimentally estimated 454/Roche (Gilles et al. 2011) and Illumina MiSeq (Quail et al. 2012) errors, respectively. Moreover, only variants which had bi-directional support of sequencing reads were further analyzed.

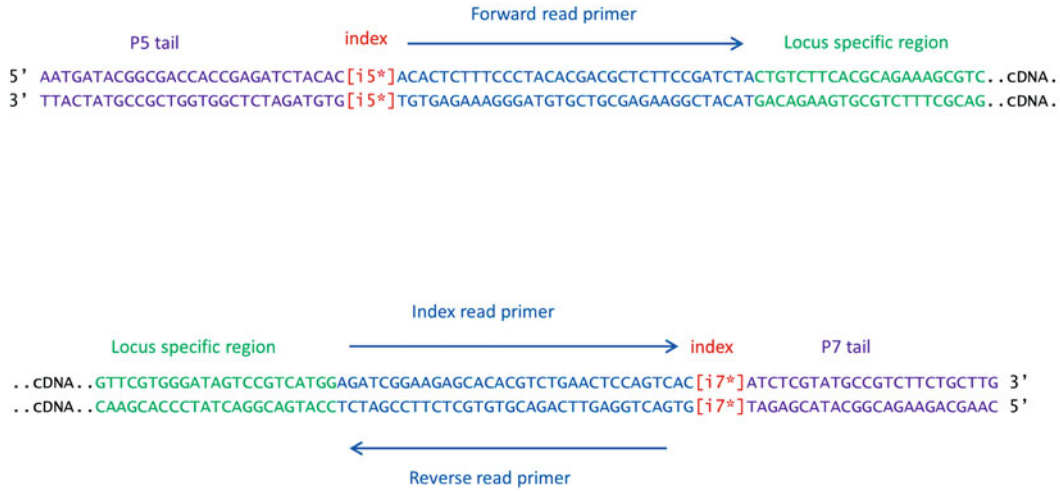

Fig. 1 Dual-index PCR primers designed for paired-end Illumina sequencing of 5'-UTR region. The P5 and P7 tails (*purple*) are used for amplicon immobilization on flow cell; indices for sample coding are in *red*; sequences complementary to Illumina sequencing primers and index read primers are in *blue*; and sequences specific to the 5'-UTR region are in *green*. The *arrows* show sequencing direction

The retained sequences of 206 bp (without primer-flanking regions) were aligned and visualized by molecular evolutionary genetics analysis (MEGA; http://www.megasoftware.net/), version 5. The phylogenetic tree was constructed according to the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). Evolutionary analyses were conducted using MEGA 5.0 software (Tamura et al. 2011). Parameters of genetic diversity were assessed by DNA SP version 5 (http://www.ub.edu/dnasp/) according to the reference sequence for the genotype 1b (GenBank: AJ242654). Thermodynamic stabilities were calculated in Mfold 3.2 program (http://mfold.rna.albany.edu) (Zuker 2003).

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993).

3 Results

3.1 Variability of 5'-UTR

By 454/Roche pyrosequencing, 21,781 sequencing reads were obtained with the mean coverage per sample of 2,349 ± 1,369 reads. After filtering, 24 different 5'-UTR variants were found (constituting 93.4 % of all reads), 8 were present in both serum and PBMC, 4 were present exclusively in the serum and 12 were found only in the PBMC samples. Complexity, as assessed from the number of genetic variants, in both serum and PBMC was similar (5.3 ± 2.3 *vs.* 5.6 ± 2.6) (Table 1).

Illumina sequencing provided 489,330 sequences (54,370 ± 39,160 per sample). After filtering, 11 different 5'-UTR variants were found (constituting 59.5 % of all reads), 5 were found in both serum and PBMC, 4 were only in the serum and 2 were found only in the PBMC samples. The mean number of variants was 4.5 ± 1.7 in the serum and 2.8 ± 1.8 in the PBMC (Table 1).

Figure 2 shows changes in the 5'-UTR complexity and nucleotide diversity *per* site. For both sequencing platforms viral complexity declined until Week 16 of treatment and then increased at Week 60 due to the recurrence of infection. Nucleotide diversity *per* site values were similar in the serum and PBMC, except for Week 16 due to the appearance of genotype 4 in the latter compartment. Baseline and post-treatment diversity values were similar.

Table 1 Characteristics of 454/Roche pyrosequencing and Illumina sequencing of HCV 5'-UTR in sequential serum and PBMC samples from a patient receiving antiviral treatment with pegylated interferon and ribavirin

	454/Roche pyrosequencing	Illumina sequencing
Sequenced reads	21,781	489,330
Sequenced nucleotides	4,486,886	100,801,980
No. of reads per sample	2,349 ± 1,369	54,370 ± 39,160
No. of genetic variants per sample after correction of sequencing errors	5.4 ± 2.0	3.6 ± 1.9
No. of genetic variants in the serum	5.3 ± 2.3	4.5 ± 1.7
No. of genetic variants in PBMC	5.6 ± 2.6	2.8 ± 1.8

PBMC peripheral blood mononuclear cells

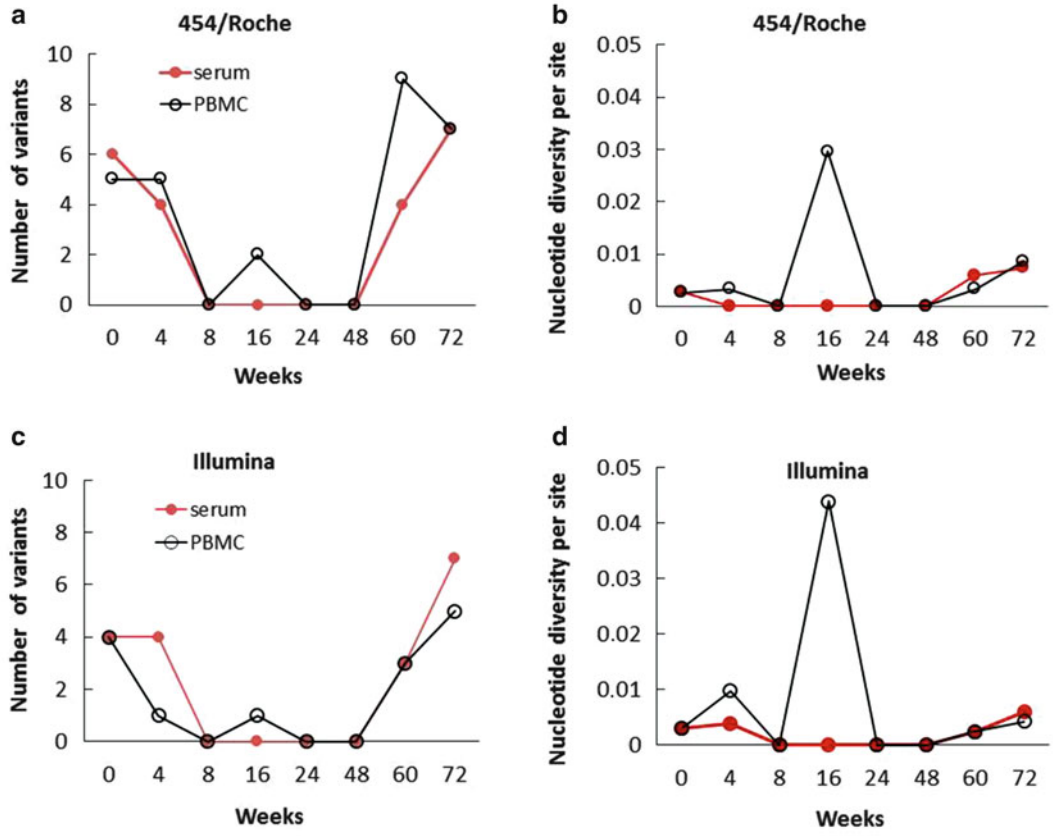

Fig. 2 Changes in genetic complexity and diversity of 5'-UTR in serum and PBMC samples before (0) during (Week 4 and 16) and after (Week 60 and 72) antiviral treatment assessed by 454/Roche (panels a and b) and Illumina (panels c and d) sequencing

3.2 Emergence of a Different Genotype

Sequence analysis revealed the presence of genotype type 4 in PBMC sample at Week 16. Two variants (R.16p_66.55 and R.16p_30.66) were identified by 454/Roche pyrosequencing, while the Illumina platform identified only one (I.16p_67.62), which corresponded to R.16p_30.66 (Fig. 3). These variants were directly responsible for the increase in diversity at Week 16 as shown in Fig. 2.

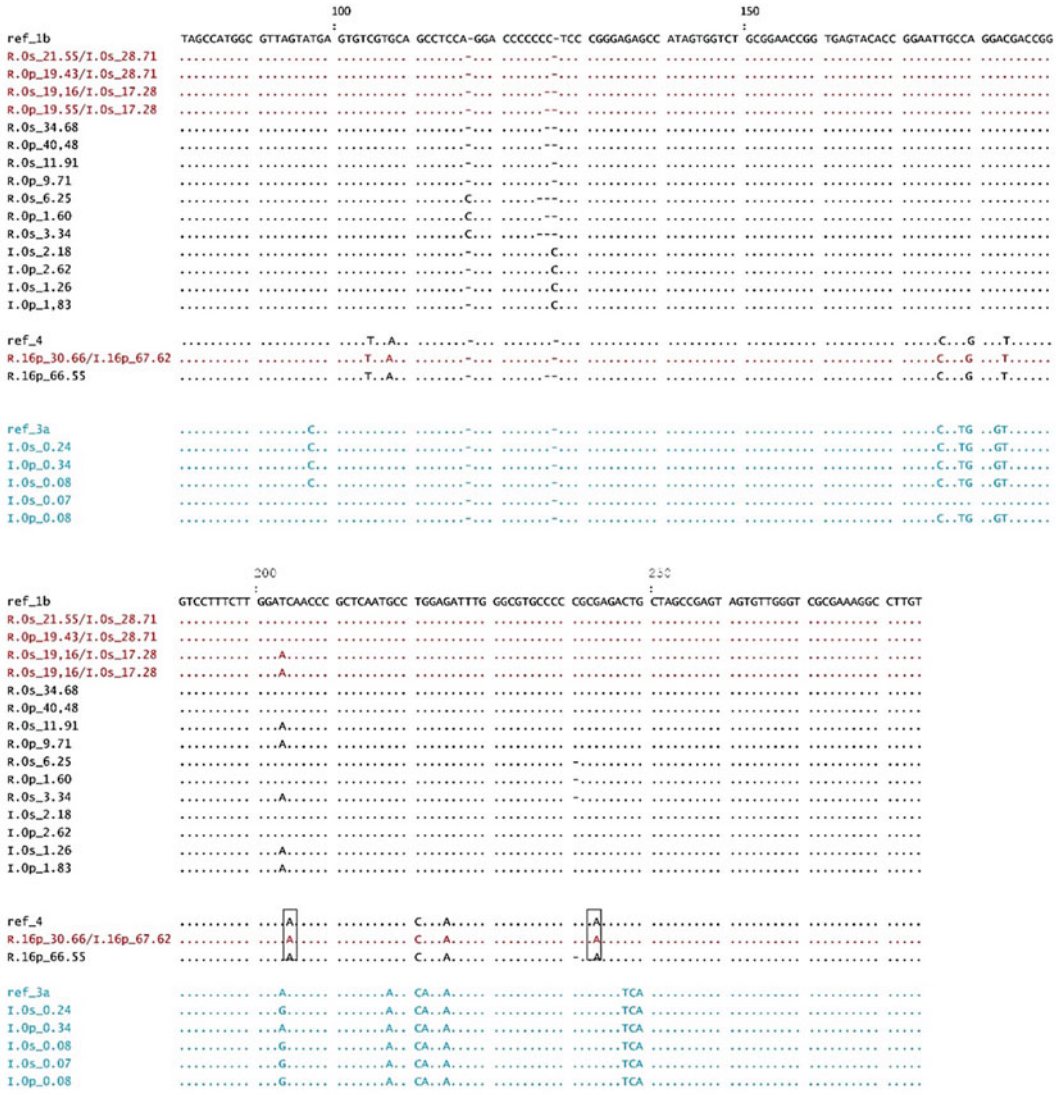

Fig. 3 5'-UTR sequences in serum (s) and PBMC (p) at baseline (0s and 0p) and at Week 16 as determined by 454/Roche pyrosequencing and Illumina sequencing platforms. Ref_1b, ref _3a, and ref_4 correspond to reference sequences of genotypes 1b (GenBank AJ242654), 3a (GenBank AF046866.1), and 4 (GenBank FJ462437), respectively. Substitutions 107A, 204A, and 243A, which were reported to be characteristic of lymphotropic HCV variants (Forton et al. 2004) are indicated by rectangular boxes. Variants identified by both sequencing platforms are marked in red, whereas sequences representing genotype 3a are marked in blue

For both sequencing platforms, viral populations in all preceding treatment and follow-up samples belonged to the same baseline type 1b genotype and no genotype 4 sequences could be found at any frequency. Interestingly, Illumina sequencing of pretreatment serum and PBMC samples revealed the presence of five HCV genotype 3a sequences at low frequencies (ranging from 0.07–0.34 %) (Fig. 3).

3.3 Phylogenetic Analysis

Four major variants which were detected by 454/Roche pyrosequencing at baseline were also present at Week 72 (Table 2). The number of minor variants (representing <4 % of the total population) was lower in the pre-treatment than post-treatment samples (2 *vs*. 6). Moreover, none of the minor baseline variants was detected in the

Table 2 5'-UTR variants, their frequencies (%), and thermodynamic stability of the secondary structure (expressed as ΔG, Gibbs free energy). Geometric marks in Fig. 5 and Table 2 correspond to each other

454/Roche pyrosequencing										
	Week 0		Week 4		Week 16	Week 60		Week 72		ΔG (kcal/mol)
Sequence	s	p	s	p	p	s	p	s	p	
●	34.7%	40.5%	56.2%			60.2%	35.7%	48.8%	40.8%	− 64.1
⌂	**21.6%**	**19.4%**	**25.9%**			**25.9%**	**17.3%**		**23.4%**	**− 64.1**
♦	**19.2%**	**19.6%**						**22.7%**	**17.2%**	**− 64.1**
◊	11.9%	9.7%						14.1%	7.5%	− 64.1
■	6.3%	1.6%	1.7%				7.8%			− 61.6
▲			3.9%				1.3%			−58.0
▼						6.3%	5.3%	1.6%	1.6%	− 61.9
□						**3.3%**	**3.3%**			**− 61.9**
	3.3%									− 61.6
				58.8%						− 64.1
○				**31.5%**						**− 64.1**
				1.7%						− 61.6
				1.6%						− 61.6
				3.7%						− 58.0
							13.7%			− 64.1
							1.2%			−58.2
							1.2%			−59.4
								3.0%		− 59.5
								1.6%		− 64.1
								1.1%		− 64.1
†									**2.8%**	**− 61.9**
									2.3%	− 61.8
					66.6%					− 64.9
□					**30.7%**					**− 64.9**
Σ	96.9%	90.8%	87.7%	97.3%	97.2%	95.7%	86.8%	92.8%	95.5%	
Illumina sequencing										
	Week 0		Week 4		Week 16	Week 60		Week 72		ΔG (kcal/mol)
Sequence	**s**	**p**	**s**	**p**	**p**	**s**	**p**	**s**	**p**	
⌂	**28.7%**	**25.6%**	**48.7%**			**54.5%**	**54.9%**	**33.0%**	**37.2%**	**−64.1**
☼	2.2%	2.6%	1.1%			2.6%	2.9%	1.7%	2.0%	−64.1
♦	**17.3%**	**12.8%**						**24.8%**	**18.4%**	**−64.1**
Δ	1.3%	1.8%						1.2%	0.8%	−64.1
□						**6.5%**	**8.9%**	**1.3%**	**2.5%**	**−61.9**
			1.0%							−63.2
			0.8%							−60.3
○				**69.7%**						**−64.1**
†								**0.8%**		**−61.9**
								0.8%		−64.1
□					**67.6%**					**−64.9**
Σ	49.5%	42.8%	51.7%	69.7%	67.6%	63.6%	66.3%	63.7%	61.0%	

Variants identified by both sequencing platforms are *bolded* out; *s* serum, *p* plasma

post-treatment period. For Illumina, similar pattern of major pre- and post-treatment (Week 72) variants was found, but the post-treatment samples contained more diverse population. In contrast to the 454/Roche pyrosequening results, two minor variants were present both in pre- and post-treatment viral populations, whereas the number of minor variants was 2 *vs*. 5. Six viral variants were detectable by both sequencing platforms (Table 2).

Phylogenetic analysis of 454/Roche pyrosequencing and Illumina reads (Fig. 4a and b) revealed close relatedness of variants present at baseline and post-treatment, whereas genotype 4 variants at Week 16 and PBMC strains at Week 4 showed marked divergence.

3.4 Thermodynamic Stability

Genotype 4 variants were characterized by the highest thermodynamic stabilities of predicted secondary structures ($\Delta G = -64.9$ kcal/mol) among all analyzed variants; (Table 2). Major strains found at pre- and post-treatment time points displayed the lowest ΔG among 1b genotype strains analyzed. However, within the particular sample populations, the most prevalent strains seemed to display the highest stability. Furthermore, only 454/Roche pyrosequencing revealed the presence of minor variants whose ΔG was >-60 kcal/mol. Interestingly, genotype 4 variants displayed the presence of mutations characteristic for immune cell tropic variants: 107A, 204A, and 243A (Fig. 3) (Forton et al. 2004).

3.5 Influence of Sequencing Platform on the Reconstructed Population of 5′-UTR

While 454/Roche pyrosequencing identified 24 different 5'-UTR variants, only 11 were identified by the Illumina platform and this difference was largely a result of variability in the two homopolymeric regions (Fig. 5). As seen, 454/Roche pyrosequencing *vs*. Illumina "sequencing generated 3 vs 2 homopolymer variants within Ist, and 2 vs 0 within IInd homopolymeric region.". Variants obtained by 454/Roche pyrosequencing were mostly made up of six repeats of C (67 %), whereas Illumina variants were made up of seven (65.6 %) repeats at the I^{st} homopolymeric region. Moreover, no eight C repeats variants were observed in 454/Roche pyrosequencing and no six and five repeats in Illumina reads. Furthermore, only 454/Roche pyrosequencing variants had mismatches at this homopolymeric region (4 %). For the II^{nd} homopolymer, only 454/Roche pyrosequencing yielded in variants with four repeats of C (24.5 %).

4 Discussion

HCV genotyping is used in clinical practice to determine the response and duration of antiviral therapy as well as for epidemiological studies (Cai et al. 2013; Tuveri et al. 1997; Horiike et al. 1996). The phenomenon of mixed infections (co-infections and superinfections with different viral genotypes or strains) may have clinical relevance as it could affect the natural course of infection, viral load, the treatment response (emergence of resistance mutations, or genotype switch), and dynamics of transmission (Blackard 2012; Blackard and Sherman 2007).

Various methods have been developed for HCV genotype determination. Most are based on amplification of specific regions (mostly 5'-UTR) and type-specific detection of sequence differences by restriction fragment length polymorphism (RFLP) analysis, line probe reverse hybridization, or sequence analysis (Buoro et al. 1999; Stuyver et al. 1995; Simmonds et al. 1994). However, these methods are only feasible for detecting a predominant genotype strain. For example, RFLP analysis is capable of detecting genotype at a level of ≥41.6 % in a mixed genotype population, whereas direct DNA sequencing had sensitivity of ≥25 %. The highest sensitivity was accomplished by Idrees et al. (2011) who used a combination of type-

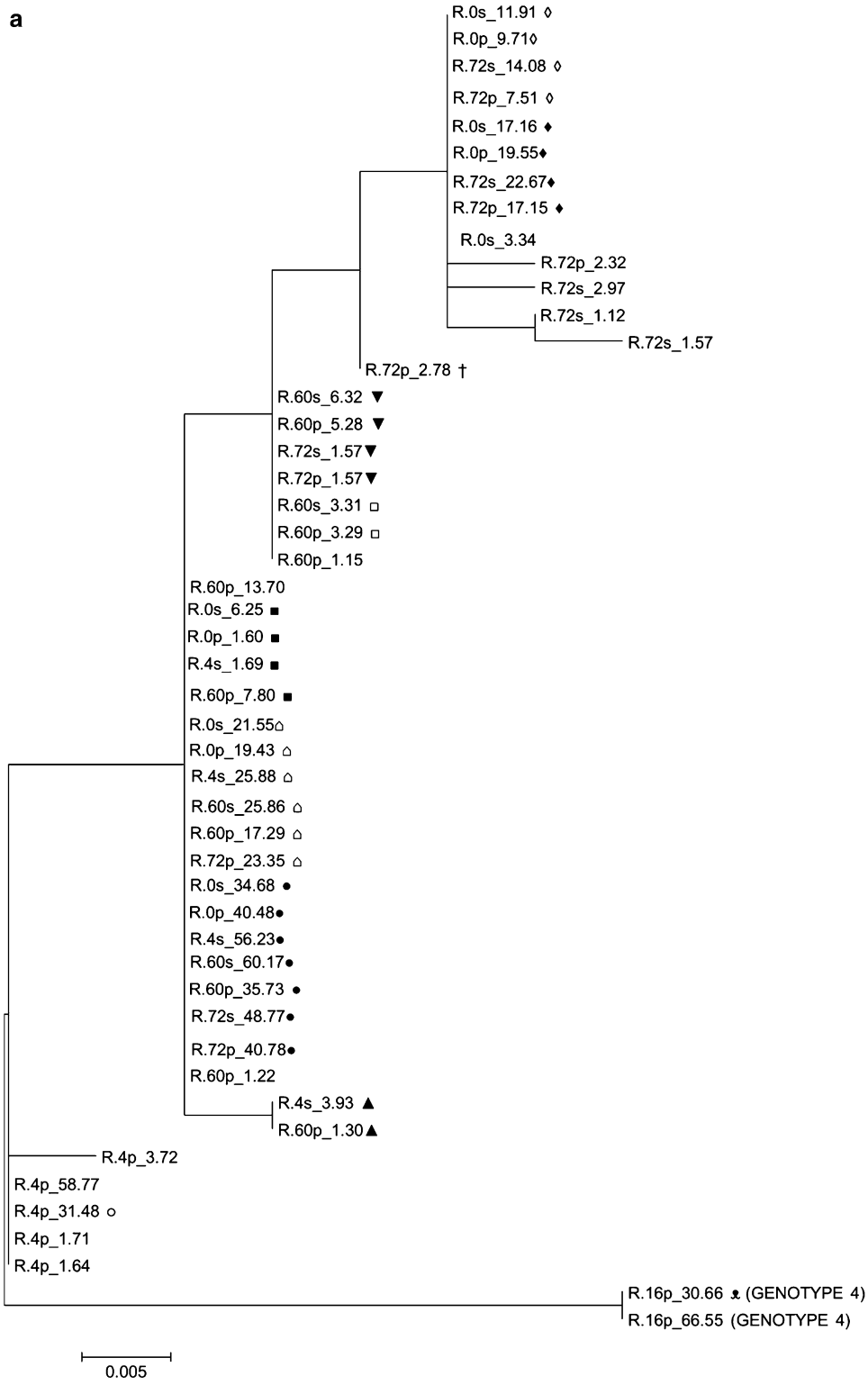

Fig. 4 Phylogenetic analysis of 5'-UTR variants present in serum (s) and PBMC (p) at baseline (0), during (Weeks 4 and, 16) and after antiviral treatment (Weeks 60 and 72). (**a**) – 454/Roche pyrosequencing – R (**b**) – Illumina sequencing – I. Numbers following underscores indicate frequencies of variants

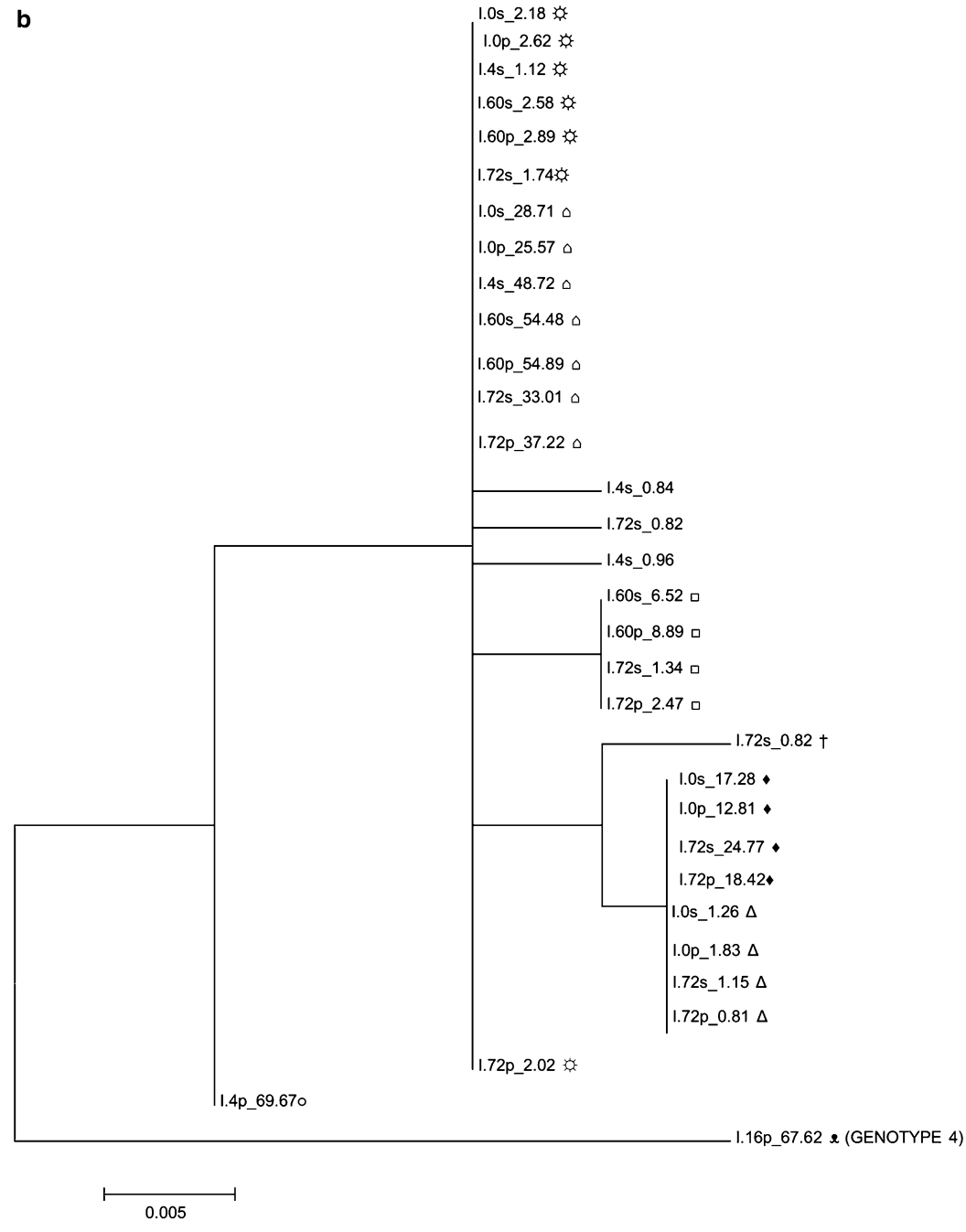

Fig. 4 (continued)

specific PCR and PAGE (polyacrylamide gel electrophoresis). That assay allowed detecting a genotype in a defined mix of HCV genotypes at a level as low as 8.3 %.

The next-generation sequencing employed in the present study offers a new approach to the detection of mixed infections. We used two different sequencing platforms, 454/Roche

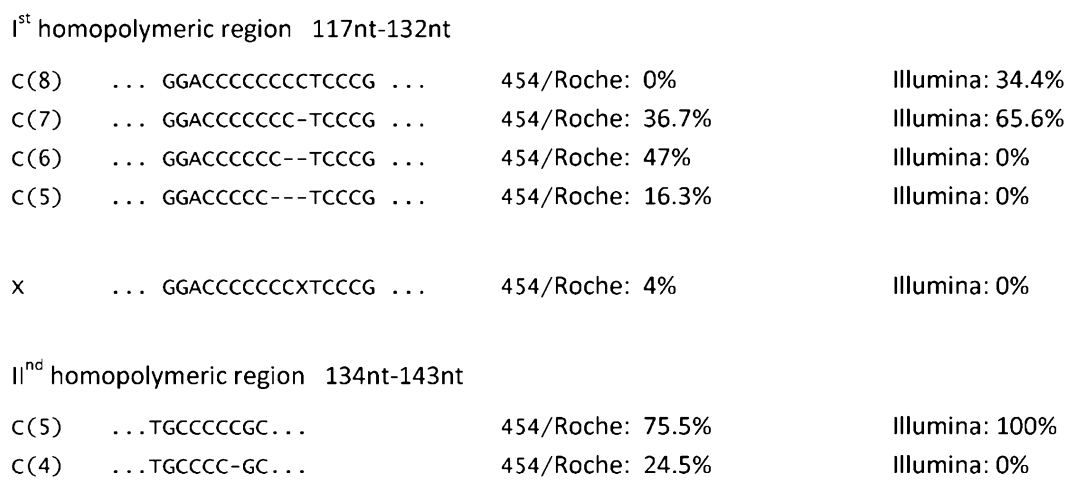

Fig. 5 Comparison of homopolymeric cytosine-rich regions variability between the two sequencing platforms as aligned to reference sequences. Numbers of repeated cytosines are indicated in brackets. Percentages reflect proportions of reads with a given characteristics; *X* – mismatch

pyrosequencing and Illumina, for a highly sensitive detection of different genotypes in a patient infected with genotype 1b, in whom genotype 4 transiently appeared during antiviral treatment. Both sequencing methods detected only genotype 4 strains in PBMC samples at Week 16. This genotype was not found in any other sample from this patient, which suggests that the observed phenomenon was due to superinfection and not to the treatment-driven selection of pre-existing strains.

A transient presence of a new HCV genotype due to superinfection has been reported in the setting of liver transplantation where both the donor and the recipient were HCV RNA positive. Superinfection may result in a rapid dominance of either the recipient or donor strain (Fan et al. 2003). Another study has estimated that the original recipient strain is retained in 30–60 % of patients (Ramirez et al. 2010).

In the present study, the superinfecting genotype 4 strain was not detected in any of the follow-up samples and this was probably due to the effect of treatment as genotype 4 is more sensitive to interferon and ribavirin than genotype 1b (Fried et al. 2002). Such a scenario is compatible with a study by Sole et al. (2002) who observed selection of genotypes 1b in two patients with genotype 2a and 3a. Interestingly, the novel superinfecting type 4 strain contained three mutations (107A, 204A, and 243A) which were previously found in monocyte-derived dendritic cells strains (Forton et al. 2004), suggesting that it was lymphotropic.

We also detected the presence of genotype 3a before treatment, but at a very low frequencies (0.07–0.34 %). The biological significance of such extremely minor variants is unclear. Importantly, the possibility of a false sample assignment, although remote, cannot be entirely excluded. This problem has been reported in previous studies and it can be caused by inter-run contamination, contamination of primers or libraries, as well as chimera formation and inaccurate demultiplexing of samples (Kircher et al. 2012). However, the double-indexing system used in the present study significantly reduces false-assignment rates when compared with the single-index sequencing (Kircher et al. 2012). Nevertheless, the possibility of mixed infection with genotype 1b and 3a before treatment cannot be excluded.

Diversity analysis of data from both sequencing platforms revealed that the post-treatment viral population was very similar to that at baseline with respect to sequence and the number of variants and their proportional distribution. Thus, once the selective pressure due to treatment

ceased, the quasi species composition returned to the original pattern implying that it was the result of a unique host-pathogen interaction.

A higher number of variants detected by the 454/Roche sequencing, as compared with the Illumina platform (24 *vs.* 11), was due mainly to differences in the sequence of homopolymeric regions. This confirms earlier observations pointing to a high number of deletions and mismatches encountered when attempting to sequence homopolymeric regions with the Illumina platform (Gilles et al. 2011). The 454/Roche pyrosequencing generated additional variants with underestimated number of C repeats when compared with Illumina variants. Those were mostly minor frequency variants, unique for particular sample. Whereas the sequences of the Ist homopolymeric region variants were present in GenBank database, there were no variants with four C repeats in the IInd homopolymeric region. As Illumina generates errors at homopolymers longer than 20 repeats (Otto et al. 2010), it seems that homopolymeric variants obtained by this platform are more credible.

In conclusion, longitudinal analysis of HCV variants, employing two independent in depth sequencing methods, suggests that the transient presence of different genotype strain in PBMC was the result of superinfection and not a selection of a pre-existing minor variant.

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

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Swelling of Erectile Nasal Tissue Induced by Human Sexual Pheromone

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Abstract

Most chemically mediated sexual communication in humans remains uncharacterized. Yet the study of sexual communication is decisive for understanding sexual behavior and evolutive mechanisms in our species. Here we provide the evidence to consider 4,16-androstadien-3-one (AND) as a man's sexual pheromone. Our experiment provides support for the physiological effect of AND on nasal airway resistance (Rna) in women, as assessed by anterior rhinomanometry. We found that AND administration increased the area of turbinate during the ovulatory phase, resulting in an increase of Rna. Thus, we discovered that minute amounts of AND, acting through neuroendocrine brain control, regulate Rna and consequently affect the sexual physiology and behavior. Fascinatingly, this finding provides the evidence of the preservation of chemosexual communication in humans, which it has been largely neglected due to its unconscious perception and concealed nature. Therefore, chemical communication is a plesiomorphic evolutive phenomenon in humans.

Keywords

Androstadienone • Human chemosexual communication • Human pheromone • Nasal airway resistance • Pheromone • Rhinomanometry • Turbinate swells

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1 Introduction

Pheromones are ectohormones, released out of the body, and are vital species-specific chemosignals that control a wide range of behavior in animals (Carluccio et al. 2013; Mazzatenta et al. 2011; Karlson and Luscher 1959). In the receiver, pheromones can trigger stereotyped innate behavior (releaser pheromones) and may stimulate long-term regulatory or developmental changes to pre-programmed neuroendocrine circuits (primer pheromones). They also can indicate the attributes of the sender to other members of the same species, such as their identity or reproductive status (signaling pheromones) or can modulate the ongoing behavior or psychophysiological reactions to a particular context (modulator pheromones) (Mazzatenta et al. 2011; McClintock 2000).

Pheromone-mediated sexual communication in humans is hitherto a controversial matter, which has likely to do with its unconscious and concealed nature and with the largely neglected function of the vomeronasal organ which is typically involved in the pheromone sensing in mammals (Carluccio et al. 2013; Keverne 2004; Wysocki and Preti 2004; Jacob et al. 2001; Meredith 2001; Monti-Bloch et al. 1998). A clear demonstration of the effects of human male pheromone has come from a study on body thermal variation induced by 4,16-androstadien-3-one (AND). In women, the effect consisted of an increase in the facial temperature in the ovulatory state, while a decrease was present in the non-ovulatory state (Mazzatenta et al. 2010). The idea that human pheromones can elicit a specific pathway activation in the limbic system, time-related to the physiological state has been reinforced in several studies (Parma et al. 2012; Wyart et al. 2007; Bensafi et al. 2003; Savic et al. 2001; Swaab et al. 2001; McClintock 2000). The activation of the hypothalamus, which controls the autonomic nervous system, is of particular interest. The autonomic nervous system is a key structure in sexual arousal, sex-hormone release, reproductive behavior, body temperature regulation, artery vasodilatation, and tissue engorgement, all of which is involved in the physiological reactions, from appetitive to consummatory reproductive behavior (Carluccio et al. 2013; Mazzatenta et al. 2010; Swaab et al. 2001). Thus, autonomic variables can vary in humans exposed to these cues. In particular, AND can induce non-conscious effects on the autonomic responses and peripheral physiological activities (Mazzatenta et al. 2010; Savic and Lindström 2008; Bensafi et al. 2003; Savic et al. 2001).

The autonomic nervous system controls the erectile tissue in the nasal cavity, which normally swells and engorges in an alternate pattern termed the nasal cycle; which, in turn, results in a differential pattern of nasal airway resistance (Rna) (Eccles 2000; Haeggström et al. 2000; Hanif et al. 2000; Hallén et al. 1996; Gilbert and Rosenwasser 1987). The Rna is measured in normal clinical practice by active anterior rhinomanometry (Eccles 2011; Shelton and Eiser 1992; Cole 1989; Kern 1981; Stoksted 1953). In the present study we investigated whether AND stimulation could affect the nasal erectile tissue in women, as assessed by Rna measurements.

2 Methods

This non-invasive and anonymous study included 11 healthy female volunteers (mean age 17.8 ± 2.7, range 15–25 years) in the ovulatory phase. The participants provided written informed consent and the procedure was performed in comport with the Ethical Standards of the Helsinki Declaration.

Active anterior rhinomanometry was performed using a Rhinospir-164 (Sibelmed, Barcelona, Spain). The 4,16-androstadien-3-one (AND) stimulus was given by inhalation (Steraloids, Newport, RI). We followed the recommendations of the International Committee for the Standardization of Rhinomanometry (Eccles 2011; Shelton and Eiser 1992; Cole 1989; Kern 1981).

After a preliminary rhinological examination, the subjects were left to acclimatize in the experimental room with a controlled temperature (23 ° C), and humidity (50–55 %) for 1 h. Nasal and turbinate images were acquired pre- and

post-AND stimulation at the same topographical position by using a turbinate spacer made *ad hoc*. Basal bilateral rhinometric measurements were acquired before stimulation, taken as an average of five breath cycles. AND was administered birhinally for 60 s followed by 5 min of recordings. Data are given as means ± SE. One-way ANOVA was the basic statistical test for all comparisons. The linear fit equation used was $y = ax + b$; α was set at 0.05. Data treatment and statistical analysis was done by Excel, Origin and SPSS software.

3 Results

There was a significant difference in pre- and post-AND Rna: 1.04 ± 0.05 Pa s/cm^3 *vs.* 1.33 ± 0.09 Pa s/cm^3 ($p < 0.05$; $F_{1.23} = 7.84$, one-way ANOVA) (Fig. 1a). The distribution of Rna values pre- *vs.* post-AND inhalation is shown in Fig. 1b, linear fits in the two conditions (pre-AND - $r^2 = 0.84$ and post-AND - $r^2 = 0.76$) were significantly different ($p < 0.001$; $F_{1.20} = 149$) and gave the intercept values of 0.73 ± 0.04 Pa s/cm^3 and 0.86 ± 0.09 Pa s/cm^3, and the slopes of 0.04 ± 0.006 and 0.075 ± 0.01, respectively.

The evaluation of laterality of the AND inhalation effects on the Rna showed no dominance of nostril function on either side ($p = 0.84$; $F_{1.44} = 0.04$), while a significant effect of AND persisted ($p < 0.05$; $F_{1.44} = 9.8$, *post-hoc* factorial ANOVA). The mean pre-AND right nostril Rna was 0.51 ± 0.04 Pa s/cm^3 and post-AND was 0.65 ± 0.05 Pa s/cm^3 ($p < 0.05$; $F_{1.23} = 5.9$) (Fig. 2a). Likewise, the left nostril Rna changed significantly after AND stimulation, 0.49 ± 0.05 Pa s/cm^3 pre- *vs.* 0.69 ± 0.07 Pa s/cm^3 post-AND ($p < 0.05$; $F_{1.23} = 4.6$) (Fig. 2b).

The area of turbinates was 3.8 ± 0.3 mm^2 pre-AND *vs.* 5.8 ± 0.2 mm^2 post-AND, and it was significantly affected by AND inhalation ($p < 0.001$; $F_{1.42} = 15.6$) (Fig. 3a). The area of airway passages was likewise affected, 4.1 ± 0.2 mm^2 pre-AND *vs.* 1.7 ± 0.2 mm^2 post-AND ($p < 0.001$; $F_{1.42} = 55.0$) (Fig. 3b).

Photomicrographs of the left and right turbinates pre- and post-AND inhalation are shown in Fig. 4. The laterality evaluation showed no difference between the two sides of the turbinate area ($p = 0.28$, $F_{1.44} = 2.0$), while there was an overall effect of AND ($p < 0.001$, $F_{1.44} = 16.0$). There was a significant effect of AND on the right (pre-AND 4.07 ± 0.6 mm^2 *vs.* post-AND ±0.3 5.5 mm^2; $p < 0.05$; $F_{1.23} = 5.0$) and left

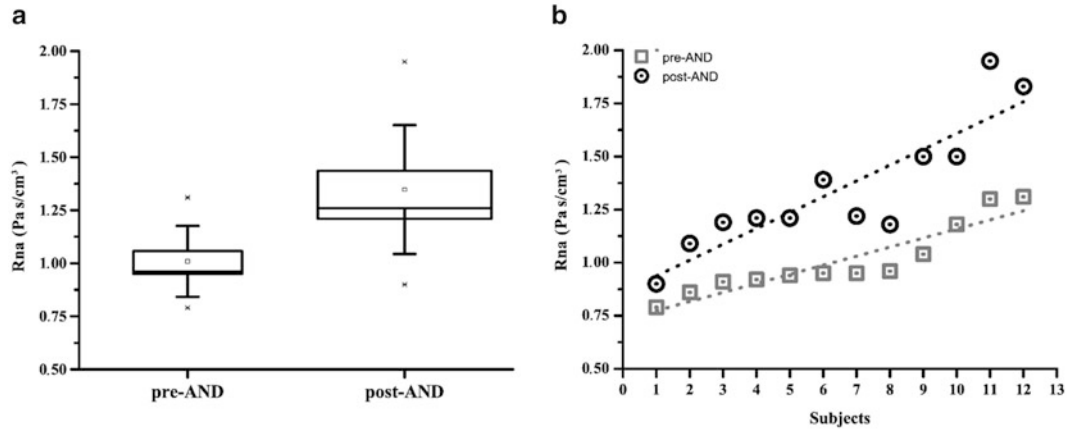

Fig. 1 (**a**) Nasal airway resistance (Rna) before and after inhalation of 4,16-androstadien-3-one (AND). *Box* and *whisker plot*: *little open squares* in the boxes are means, *bars* are SD bars, the *hight of boxes* corresponds to SE, *horizontal lines* in the boxes are medians, 'x' denote maxima and minima; $p < 0.0001$ for pre- *vs.* post-AND. (**b**) Comparison of average Rna variations pre- and post-AND inhalation in the same subject. *Dotted lines* are linear regression fits through each set of data; $p < 0.05$ for the difference between the two slopes

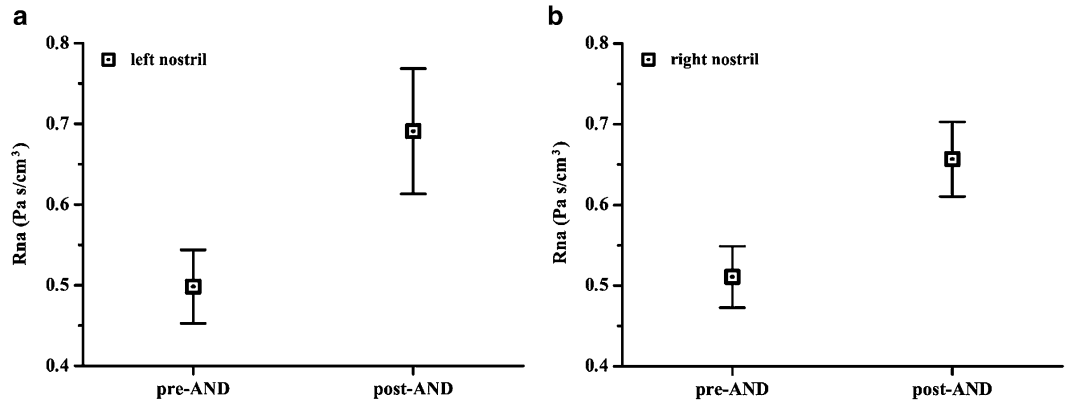

Fig. 2 Nasal airway resistance (Rna) before and after inhalation of 4,16-androstadien-3-one (AND) in *left* (**a**) and *right* (**b**) nostril. AND caused significant enhancement of Rna in each nostril; $p < 0.05$. Bars are SE bars

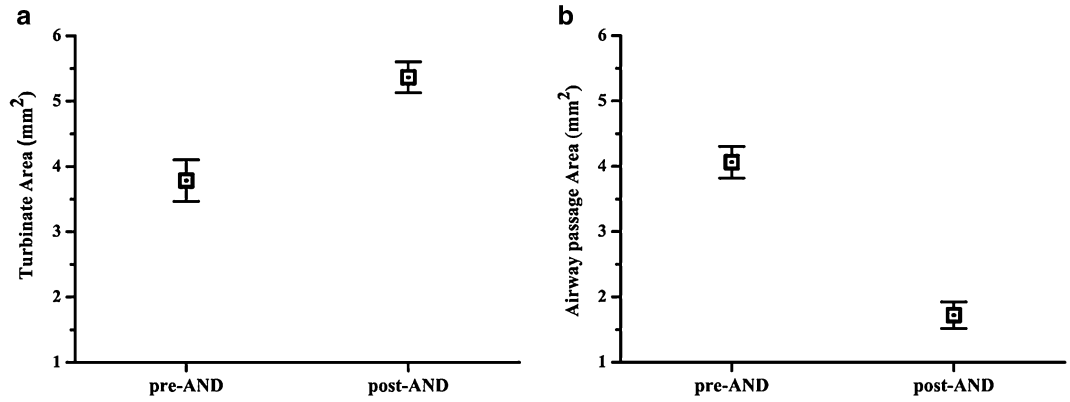

Fig. 3 (**a**) Area measurement in the anterior part of the left inferior turbinate and (**b**) Relative airway passage before and after inhalation of 4,16-androstadien-3-one (AND). AND caused tissue swelling which significantly congested the naris; $p < 0.001$. Bars are SE bars

(pre-AND 3.5 ± 0.3 mm^2 *vs.* post-AND 5.2 ± 0.3 mm^2; $p < 0.05$; $F_{1.23} = 14.0$) turbinate area, pointing to a massive congestion of turbinates.

4 Discussion

In the present study we measured nasal airway resistance, as represented by slope and intercept of the distribution, after administration of the putative human pheromone AND by way of inhalation in a sample of female subjects. AND distinctly increased nasal airway resistance. We demonstrated that the increase in nasal resistance recorded after AND was not due to a physiological cycling of nasal pattern, since for the analysis of the laterality of changes we took the data of at least two complete nasal cycles. In fact, we did not find any functional laterality effect, but only the AND stimulation effect. Assessing the anterior part of the inferior turbinate and the space surrounding it, we found an increase in the turbinate dimension induced by AND. Consequently, there was a decrease in the nasal air passage, with a resulting increase in nasal airway resistance.

The issue of the role of human pheromone has been discussed since its discovery. Pheromones are well accepted as part of animal communication, but in case of man, the issue is unsettled and controversial (Keverne 2004; Wysocki and Preti 2004; Jacob et al. 2001; Meredith 2001; Monti-Bloch et al. 1998). Demonstrations of several

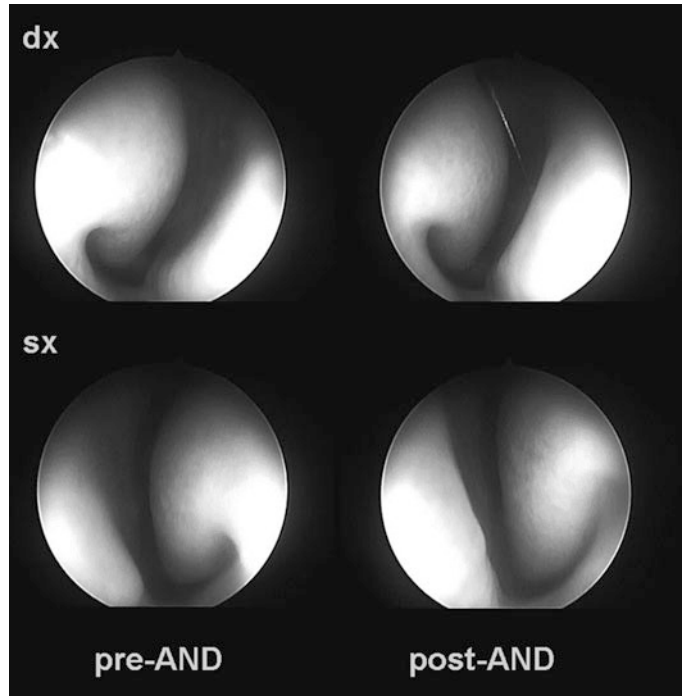

Fig. 4 Representative photomicrograph of the *right* (dx) and *left* (sx) turbinates before and after inhalation of 4,16-androstadien-3-one (AND). Note variation in the airway passage and turbinate dimension

behavioral (Parma et al. 2012; Jacob et al. 2001) and physiological effects (Wyart et al. 2007; Bensafi et al. 2003; McClintock 2000), and of brain areas stimulated by these compounds in human subjects (Savic and Lindström 2008; Savic et al. 2001) gave a momentum for further discussions, but has not settled the doubts surrounding the issue. The present findings, similarly to an earlier work by Mazzatenta et al. (2010), show that pheromones may transmit physiological and behavioral message in humans by way of the flow of chemosignals. In brief, we found that nasal AND inhalation in women being in the ovulatory phase induces engorgement of the nasal erectile tissue, measured as increases in both nasal resistance and turbinate area. These physiological effects could point to the mechanisms of the pheromone role in the reproductive behavior. Pheromones may elicit the engorgement of genitals. Furthermore, there is another intriguing aspect regarding the respiratory and cardiac effects of pheromones. The nasal tissue swelling, increasing nasal resistance, may cause a decline in arterial oxygen level, particularly if associated with increased metabolic activity. Thus, there might be a pheromone-physical activity-hypoxia link that would participate in the regulation of physiological and behavioral aspects of reproduction. This issue has obviously to do with aspects of the human sexual behavior. The compound exerts bodily effects through the autonomic nervous system, which seems independent of its sensing mechanism.

In conclusion, we believe we have demonstrated that AND acts as a human pheromone. It produces behavioral consequences that have to do with the chemosexual communication in humans.

Competing interests The authors declare no competing interests in relation to this article.

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Influenza Vaccination Coverage Rate for Medical Staff: Influence of Hospital-Based Vaccination Campaign

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Abstract

Despite intensive recommendations, influenza vaccination rate in medical staff in Poland ranges from about 20 % in physicians to 10 % in nurses. The objective of this work was to assess the influence of hospital influenza vaccination campaign directed toward health care workers, combined with dispensing free of charge vaccine, on vaccination rate. The campaign was conducted by the Hospital Infection Control Team of the Czerniakowski Hospital in Warsaw, Poland, separately for physicians, nurses, and physiotherapists. Overall, 37 % of medical staff were vaccinated, including 55 % of physicians and 21 % of nurses. Concerning physicians, the greatest vaccination rate was in the orthopedic (80 %) and ophthalmology units (73 %), whereas the lowest rate was in the intensive care (22 %) and neurology units (20 %). Concerning nurses, the greatest vaccination rate was in those working in the outpatient (40 %) and emergency units (29 %), whereas the lowest rate was in the ophthalmology (6 %) and surgery units (11 %). We conclude that the professional knowledge campaign combined with the incentive of free of charge vaccine substantially raises the vaccination rate among medical staff.

Keywords

Health care workers • Influenza • Nurses • Physicians • Vaccination • Education

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1 Introduction

Influenza is still one of the most important medical problems. Every year millions of people become infected. Three influenza pandemics occurred in the twentieth century: in 1918–1919, 1957–1958, and 1968–1969 and caused millions of deaths (Doshi 2008). Influenza is most dangerous for children, elderly people, and patients with chronic diseases. The single best way to prevent seasonal flu is to get vaccinated each year (CDC 2014). The annual influenza vaccination is recommended not only for patients but also for health care workers, such as physicians, nurses, or physiotherapists. According to CDC (2014) and ECDC (2014) recommendations, health care workers should be regularly vaccinated against influenza to reduce the morbidity associated with influenza in healthcare settings. It has been demonstrated that vaccination of health care workers against influenza significantly decreases mortality of elderly people remaining under the long-term care (Carman et al. 2000). In the opinion of CDC (2014), a high percentage of medical staff vaccinated against influenza speaks for the safety of medical institution. Hence, the indication to vaccinate medical staff against influenza has both ethical and medical considerations. Vaccinating health care workers also substantially reduces sickness absenteeism, especially in emergency units (Chan 2007).

Despite intensive recommendations, a share of medical personnel regularly vaccinated against influenza remains very low in many European countries (ECDC 2014). We have previously found that 22.3 % of physicians, 10.6 % of nurses, and 13.4 % of medical students get vaccinated against influenza (Zielonka et al. 2009). In reality, a considerably fewer medical staff may be vaccinated at local establishments. In the Czerniakowski Hospital in the capital city of Warsaw, Poland, in which the present study was conducted, according to the Ministry of Health data, 13 (2 %) staffers were vaccinated in the 2009/2010 season and 34 (6 %) in the 2010/2011 season. Concerning physicians, vaccination avoidance stemmed from the lack of knowledge of protective value of vaccine (33 %), lack of time to get vaccinated (29 %), and Laziness (24 %). In nurses, these figures amounted to 55 %, 12 %, and 5 %, respectively (Zielonka et al. 2009). In addition, 10 % of physicians and 17 % of nurses pointed to the cost of vaccine as the liming factor in their decision to avoid vaccination. Therefore, the objective of the present study was to assess the influence of hospital influenza vaccination campaign directed toward health care workers, combined with dispensing free of charge vaccine, on vaccination coverage rate.

2 Methods

The study was conducted in accordance with the 2013 revision of Declaration of Helsinki of the World Medical Association and was approved a local Ethics Committee. Participation in the study was voluntary. The survey was conducted in the Czerniakowski Hospital in Warsaw, Poland, a second level hospital, with 150 physicians, 241 nurses, 51 medical secretaries, and 105 members of technical personnel, such as radiological, electrocardiology technicians, physiotherapists, and others. The vaccination campaign was conducted by the Czerniakowski Hospital Infection Control Team between October 2011 and January 2012. Before vaccination onset, an educational campaign was carried out at the entire hospital. The campaign was separately directed to physicians, nurses, and physiotherapists. An educative session (taking 20–30 min) was conducted by a doctor expert in the vaccination area. He described the risks of influenza and its complications for both health care workers and their patients. Details regarding the safety and effectiveness of influenza vaccination were also provided during seminars. It was emphasised that the vaccination is recommended for all medical workers who have a direct contact with patients, especially with young children, elderly people, and patients with immunological deficits. Contraindications and adverse reactions

to influenza vaccine were also tackled. Special attention was drawn to ethical aspects of vaccination against influenza among health care workers. Participants of the seminars had the opportunity to ask the lecturer about all problems concerning influenza as a disease, vaccination against it, and the vaccination campaign at the hospital. The majority of questions asked concerned indications for vaccination and adverse reactions to vaccination.

The educational campaign was conducted in all hospital units, and was linked with the distribution of free of charge vaccination at the work place. Overall, 24 seminars were conducted; 13 for nurses and orderlies, 10 for physicians, and 1 for physiotherapists; the staff having close contact with patients. The administrative staff was not a target of this vaccination campaign. Seminars for physicians were conducted during traditional morning meetings of physicians with the head of a unit. Seminars for nurses were set up separately due to their shift work.

The appointment for influenza vaccination was individually arranged with each health care worker. The vaccination was voluntary and free of charge (reimbursed by the hospital management). All health care workers received a vaccine against the antigens A/California/7/2009 (H1N1), A/Perth/16/2009 (H3N2), and B/Brisbane/60/2008. Medical qualification for vaccination, in the form of a questionnaire to be filled out by a person, was given 24 h before vaccination by a physician from the Czerniakowski Hospital Infection Control Team. The questionnaire contained questions concerning demographic data and medical information related to the potential contraindications for vaccination, such as allergy, current infections, previous vaccinations, and adverse reactions.

3 Results

During the professional knowledge campaign on influenza vaccination, 158 (33 %) health care workers were vaccinated by the Hospital Infection Control Team. In addition, 19 other workers reported they were vaccinated independently outside the hospital. Overall, 177 health care workers (37 %) were vaccinated against influenza. Among the vaccinees, physicians comprised the biggest group – 74 persons, followed by nurses – 45 persons. Moreover, 14 orderlies, 10 kitchen workers, 8 members of technical personnel, 4 physiotherapists, and 4 medical secretaries were vaccinated. The highest proportion of vaccinated physicians was in the orthopaedic (80 %) and ophthalmology units (73 %). In contrast, the lowest proportion of the vaccinated physicians was in the intensive care (22 %) and neurology units (20 %) (Table 1). The highest influenza vaccine coverage rate was among nurses working at the outpatient (40 %) and emergency units (29 %), whereas the lowest rate was reported at the ophthalmology (6 %) and surgery units (11 %) (Table 2).

Two hundred and fourteen employees participated in the training: 104 physicians, 82 nurses, 18 orderlies, and 10 physiotherapists. Concerning the physicians, a majority of those working in the hospital (69 %) participated in the training (from 36 % in the otolaryngology to 100 % in the neurology unit) (Table 3). In the majority of hospital units, more physicians were vaccinated after participation in the knowledge seminars compared with the number of physicians vaccinated without having such seminars. The only exception was the surgical unit where there was no tangible influence of participating in the seminars on a decision to be vaccinated. Also, in the majority of hospital units the percentage of the non-vaccinated physicians among those who participated in the educational seminars was lower than that of the non-vaccinated physicians among those who did not participate in the seminars. However, a positive effect of gaining knowledge was not demonstrated in the non-vaccinated physicians in the intensive care, neurology, and surgery units (Table 3).

The percentage of nurses participating in the knowledge seminars on influenza vaccination was lower compared with that of physicians, just 33 %. The effect of seminars also was

Table 1 Number and proportion of hospital physicians vaccinated against influenza

Hospital units	No. of physicians	No. of physicians vaccinated in hospital	No. of physicians vaccinated outside hospital	Percentage of all vaccinated physicians
Surgery	20	10	1	55 %
Orthopaedic	15	10	2	80 %
Otolaryngology	26	14	0	54 %
Ophthalmology	11	8	0	73 %
Neurology	10	1	1	20 %
Internal medicine	48	26	2	58 %
Intensive care unit (ICU)	9	1	1	22 %
Emergency	2	0	1	50 %
Outpatient clinic	10	4	1	50 %
Overall	151	74	9	55 %

Table 2 Number and proportion of hospital nurses vaccinated against influenza

Hospital units	No. of nurses	No. of nurses vaccinated in hospital	No. of nurses vaccinated outside hospital	Percentage of all vaccinated nurses
Surgery	37	4	0	11 %
Orthopaedic	26	5	1	23 %
Otolaryngology	33	8	0	24 %
Ophthalmology	17	1	0	6 %
Neurology	21	4	0	19 %
Internal medicine	40	8	2	25 %
ICU	31	3	1	13 %
Emergency	21	4	2	29 %
Outpatient clinic	15	6	0	40 %
Overall	241	45	6	21 %

ICU intensive care unit

different in this group. In the majority of hospital units, except the ophthalmology, intensive care, and outpatient units, the percentage of vaccinated nurses among those who participated in the knowledge seminars was lower than that of vaccinated nurses among those who did not participate in the seminars. However, the percentage of non-vaccinated nurses among those who participated in the seminars was again lower than that of the non-vaccinated nurses who did not participate in the seminars (Table 4). The lack of a positive effect on vaccination frequency of knowledge seminars in nurses was noted only in the intensive care and orthopaedic units.

4 Discussion

The findings of the present study point to the efficiency of a more selective means of transmission of knowledge on influenza in terms of vaccination rate among health care workers. Similar observations have previously been made in Singapore (Lee and Fong 2007) and Brazil (Takayanagi et al. 2007), where appropriate education of medical staff contributed to the increase in a percentage of personnel vaccinated against influenza up to 66 % and 34 %, respectively. Also, in the US similar educational activity had a significant influence on the growth of

Table 3 Proportion of vaccinated and unvaccinated physicians depending on participation in the knowledge seminars

Hospital units	Participants of seminars	Vaccinated after seminars	Vaccinated without seminars	Unvaccinated after seminars	Unvaccinated without seminars
Surgery	75 %	33 %	45 %	63 %	0 %
Orthopaedic	73 %	82 %	27 %	18 %	25 %
Otolaryngology	62 %	56 %	36 %	44 %	50 %
Ophthalmology	36 %	75 %	63 %	25 %	29 %
Neurology	100 %	20 %	0 %	80 %	0 %
Internal medicine	50 %	63 %	27 %	37 %	35 %
ICU + Emergency	54 %	33 %	33 %	67 %	63 %
Outpatient clinic	50 %	75 %	33 %	25 %	83 %
Physiotherapists	91 %	40 %	0 %	60 %	100 %

ICU intensive care unit

Table 4 Proportion of vaccinated and unvaccinated nurses depending on participation in the knowledge seminars

Hospital units	Participants of seminars	Vaccinated after seminars	Vaccinated without seminars	Unvaccinated after seminars	Unvaccinated without seminars
Surgery	19 %	29 %	50 %	71 %	83 %
Orthopaedic	27 %	14 %	83 %	86 %	70 %
Otolaryngology	23 %	29 %	75 %	74 %	77 %
Ophthalmology	47 %	13 %	0 %	87 %	56 %
Neurology	24 %	40 %	50 %	60 %	82 %
Internal medicine	28 %	36 %	60 %	64 %	80 %
ICU	48 %	27 %	0 %	73 %	59 %
Emergency	53 %	25 %	67 %	75 %	77 %
Outpatient clinic	53 %	50 %	33 %	50 %	77 %

ICU intensive care unit

vaccination rates among health care workers (Prematunge et al. 2012).

The knowledge seminars were just a reminder and a review of issues related to vaccination against influenza and not the main source of information. The questions asked and opinions expressed by physicians or nurses on vaccinations showed that their knowledge in this area was far from the current evidence-based medicine recommendations. Nurses, in particular, commonly presented opinions similar to those which can be found in anti-vaccination movements and forums. That demonstrates that the education of nurses regarding prophylaxis of infectious diseases, including influenza, is ineffective. It seems essential to come to know the underlying causes of this phenomenon to work out an appropriate strategy for improvement. It is necessary not only to draw attention to medical aspects, but also to ethical, legal, and economical aspects of missing vaccination on the population, families, and patients alike (Poland et al. 2005).

The attitude of physicians toward influenza vaccination vary greatly. In many a ward, a majority of physicians were vaccinated (70–80 %). However, in the neurology and intensive care units the proportion of vaccinated physicians amounted only to 20 %. The reason for such a small yield of vaccinees was a critical opinion about the effectiveness and safety of vaccination. Similar differences, depending on medical specialty, were observed in Germany (4–71% of vaccines) (Roggendorf et al. 2011). In the US, the most frequently vaccinated physicians were pediatricians (84 %) and the least frequently were surgeons (43 %) (Christini

et al. 2007). Perhaps, the specialty-related differences in the influenza vaccination rate have to do with differences in education. Pediatricians have the highest knowledge and experience in the domain of vaccination, not only in the US but also in European countries (Tapiainen et al. 2005). Neurologists may fear neurological complications after influenza vaccination (de Wals et al. 2012). It is difficult to explain the fear of influenza vaccination among the staff of intensive care units, since these are exactly the units where many patients with most severe cases of influenza are admitted and often die (Ayscue et al. 2014). In this group of health care workers, high efficiency of influenza vaccination has been clearly demonstrated (Apisarnthanarak et al. 2010).

In the present study a strong difference between the proportion of vaccinated physicians (55 %) and nurses (21 %) was demonstrated, which is in line with some data coming from other countries. In the US, 69 % of physicians and 46 % of nurses get a vaccine shot (Christini et al. 2007), and in Germany the respective percentages are 39 % and 17 % (Wicker et al. 2009). In Italy, physicians are twice as frequently vaccinated as are nurses (Esposito et al. 2008). However, a different situation is observed in Asia. In China, 21 % of nurses and only 13 % of physicians are vaccinated against influenza (Seale et al. 2010a), and in Korea, 91 % and 68 % respectively (Lee et al. 2008). These differences can be explained by cultural and educational factors.

The assurance of free of charge influenza vaccination for health care workers was an important factor in this campaign. However, the cost of vaccine should not be overestimated as an antimotivator for vaccination among health care workers. Two years earlier in the same hospital, only 13 health care workers decided to become vaccinated during a free of charge influenza vaccination campaign. Nonetheless, a free of charge influenza vaccination for health care workers is recommended by CDC (2014), but in Poland it is not realized due to economic reasons. It is not possible to improve the influenza vaccine coverage rate among nurses and orderlies without a guarantee of free of charge vaccination, since the cost is an important argument against vaccination for these groups of medical staff (Zielonka et al. 2009).

Another essential element of the present campaign was vaccination at the workplace, which maximally shortens the time that must be taken out for the vaccination procedure. Lack of time was frequently raised by physicians as a cause not getting vaccinated (Zielonka et al. 2009). Among physicians the results were very good because even 80 % were vaccinated in some wards. That confirms the importance of setting up vaccination against influenza on-site. A good effect of vaccination of health care workers on-site also was observed by other authors (Lee and Fong 2007).

Thanks to the knowledge campaign, the influenza vaccination coverage rate among health care workers increased from 2–6% in 2009–2010 to 37 % in 2011. Our results are better than those in many European countries, for example 29 % in Germany (Roggendorf et al. 2011), 26 % in France (Guthmann et al. 2012), 20 % in Italy (Esposito et al. 2008), 16 % in Greece (Maltezou et al. 2007), 24 % in Turkey (Torun and Torun 2010), 22 % in Australia (Seale et al. 2010a), and 18 % in China (Seale et al. 2010b). The difference could be even greater, since in the articles quoted, the source of data was a questionnaire filled out by health care workers, which is might be involved with the inherent biases in providing true information in self-reporting methods. The present results come from a list of identified vaccinated persons against the list of all hospital employees. Nonetheless, the coverage rate of the Polish medical staff remains to be improved considering the 67 % coverage in the US during the 2011–2012 season (Lu et al. 2013) or 84 % of physicians vaccinated A(H1N1) virus in 2011 in Canada (Nowrouzi-Kia and McGeer 2014).

In conclusion, appropriate education combined with a free of charge vaccination offered at the work place may significantly increase the influenza vaccine coverage rate among health care workers. The issue of how to increase the coverage rate for influenza vaccination among

health care workers should be subject to knowledge campaigns that take into consideration multifaceted motivational aspects.

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

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Relationships Between Tobacco Abuse and Self-Assessment of Health

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Abstract

Smoking cigarettes negatively influences the functioning of the body. Among other effects, it has an important impact on the respiratory system, circulation, and behavior. It leads to morphological and physiological changes in organs and tissues, so it can change mood. The aim of this study was to assess the relationships between tobacco abuse and self-assessment of health. The survey was conducted among Polish (243) and foreign (80) medical students at the Pomeranian Medical University in Szczecin, Poland. The study was based on a survey questionnaire of the authors' own design, comprising open and multi-choice questions. Our questionnaire was based on the international standard questionnaire from the Health Behavior in School-Aged Children study (Currie et al. 2009). 80 % of students surveyed were free of any chronic diseases. The results showed that only 23 % of the women and 20 % of the men assessed their health as very good, over 60 % as good, and the remaining at lower levels. We did not observe significant differences between smokers and non-smokers. Physical activity in both groups was generally assessed as good or sufficient. We did not observe significant differences between groups in the incidence of headache, abdominal pain, or vertigo. Significant differences were found in the intake of painkillers.

Keywords

Cigarette smoking • Health promotion • Low regulations • Respiratory diseases • Tobacco abuse

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1 Introduction

The World Health Organization (WHO) defined health in 1946 as "a state of complete physical, mental, and social well-being, and not merely the absence of disease or infirmity" (World Health Organization 2006). This understanding of health is shaped by many factors, but lifestyle is one of the most important of them (Gawlikowska-Sroka et al. 2009, 2013). Studies on the lifestyle of children and adolescents conducted systematically in Europe show the significance of dependence on tobacco, alcohol and drugs, reduced physical fitness, and self-assessment in this population (Wojnarowska et al. 2008; Currie et al. 2009). There is also a need for continuous educational and prophylactic activities, supported by legislative measures to limit the use and availability of psychoactive substances (Crone et al. 2003; Kwapisz and Głowacka 2005). In many countries, enforced legislation limiting smoking in public places has contributed directly to a reduction in passive smoking, and in many cases has reduced the number of active smokers (Gawlikowska-Sroka et al. 2013; Abu Shomar et al. 2014). In a long-term policy these measures will be important for limiting the negative effects of smoking and reducing the incidence of diseases closely associated with smoking; therefore improving the quality of life and health of populations.

Contemporary studies on the multifactorial assessment of health and quality of life of patients attribute a significant role to the assessment of self-reported factors (Currie et al. 2009; Szkultecka-Dębek and Mazur 2005; Health interview survey 1996). The assessment of these factors enables the description of health and well-being from the perspective of the patient, and may differ from the parallel assessment made by doctors. Studies demonstrate a close relationship between self-assessment of health by adolescents and risk behaviors (Brener et al. 2003; Crone et al. 2003). Adolescents often are unaware of the consequences of such behaviors (Rosewich et al. 2008). Analyses carried out in the United Kingdom in a group of older patients revealed a close relationship between self-assessment of health and life expectancy. Usually, older subjects who presented with unhealthy behaviors, including cigarette smoking or low level of physical activity, have lower self-assessment. These subjects also present with pronounced symptoms of circulatory or respiratory disorders, and are highly aware of the impact of nicotine on human body.

The aim of this study was to analyze the relationship between cigarette smoking and self-assessment of health in young people who may not yet have experienced the consequences of smoking but should be aware of such consequences due to their chosen medical profession.

2 Methods

The study was performed in conformity with the Declaration of Helsinki for Human Research and according to the regulations of a local Ethics Committee. The protocol consisted of the analysis of the behavior of Polish students of Medical and Dentistry Faculties, as well as the first-year of foreign students of the English Program at the Pomeranian Medical University in the city of Szczecin, Poland. It was a self-reported survey completed by Polish (66 males, 177 females) and foreign medical students (28 males, 52 females). The foreign students were mainly Norwegian, while a few of them came also from Sweden and Germany. A survey questionnaire of the authors' own design was composed of open and multi-choice questions about the prevalence of smoking, level of physical activity, and a self-assessment of health. Our questionnaire was prepared on the basis of the international standard questionnaire from the Health Behavior in School-Aged Children study (HBSC) (Currie et al. 2009), General Heath Questionnaire Scale (GHQ-12) (Goldberg and Hillier 1979), and Student Life Satisfaction Scale (Huebner 1991).

All parts of the survey questions concerning the health self-assessment were constructed in the way that the respondents could only indicate the proposed variants of the response, so that the

results are shown in multi-way tables. The significance of differences in the frequency of different variants of answers for smokers and non-smokers was assessed using Pearson's Chi^2 test. Yates' correction was used in cases where the number of observed cases was small. A p-value <0.05 was used to define statistical significance of differences.

3 Results

The survey results reveal that about 40 % of students were regular or occasional smokers (Fig. 1). There were significantly fewer smoking foreign women than smokers in the other groups ($p = 0.001$). No significant differences were observed among the Polish women, Polish men, and foreign men.

Students declaring themselves to be regular smokers smoked about 10 cigarettes per day. We found no significant differences in the number of smoked cigarettes between women and men or between Polish and foreign students. We also analyzed the level of physical activity as a measure of active contribution to maintaining good health. Most of the surveyed students assessed their own physical fitness as good. However, foreign smoking female students defined their physical fitness as bad significantly more often than the students of all the other groups ($p < 0.01$) (Fig. 2). Also, more detailed analysis revealed that the time spent on exercising was shorter than recommended. Only did the Polish students spend more than the recommended one hour daily on physical activity. In the other groups, the time spent on exercise was insufficient.

Next, the students were asked to self-assess their health. Most respondents, both male and female, assessed the health as good or very good. The Chi-squared test demonstrated no significant differences in the percentage of answers among the non-smoking and smoking students, each group pooled together irrespective of gender and nationality. The only exception were the smoking students who rated their health as bad, and who significantly prevailed over the non-smokers with a bad perception of health ($p < 0.01$) (Fig. 3). In the group of smoking students as many as 37.5 % of foreign respondents assessed their health as sufficient. Most of them did not suffer from chronic diseases and declared themselves not to have asthma, allergies, eating disorders, circulatory diseases, abdominal pain, headache, vertigo, or back pain. We found no significant differences in the incidence of chronic diseases between the analyzed groups. However, the intake of painkillers indicated significant differences in

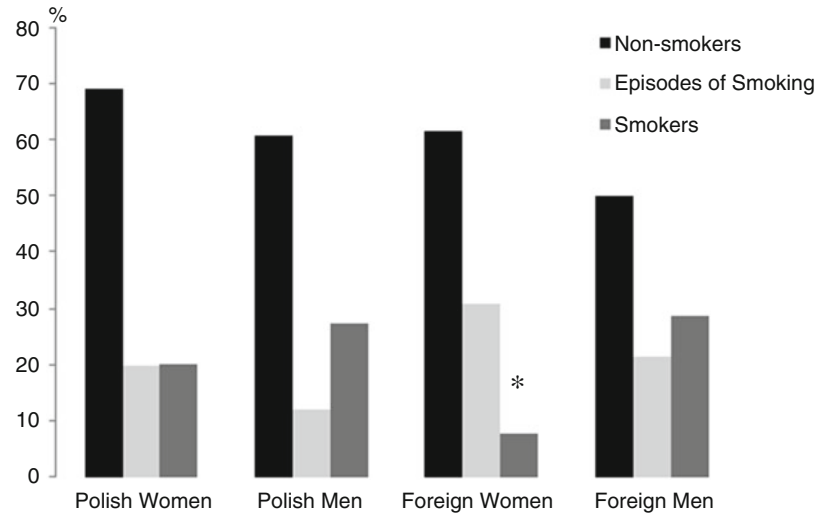

Fig. 1 Tobacco addiction among medical students (*$p < 0.001$ denotes significantly fewer smoking foreign women compared with the number of smokers in the other groups)

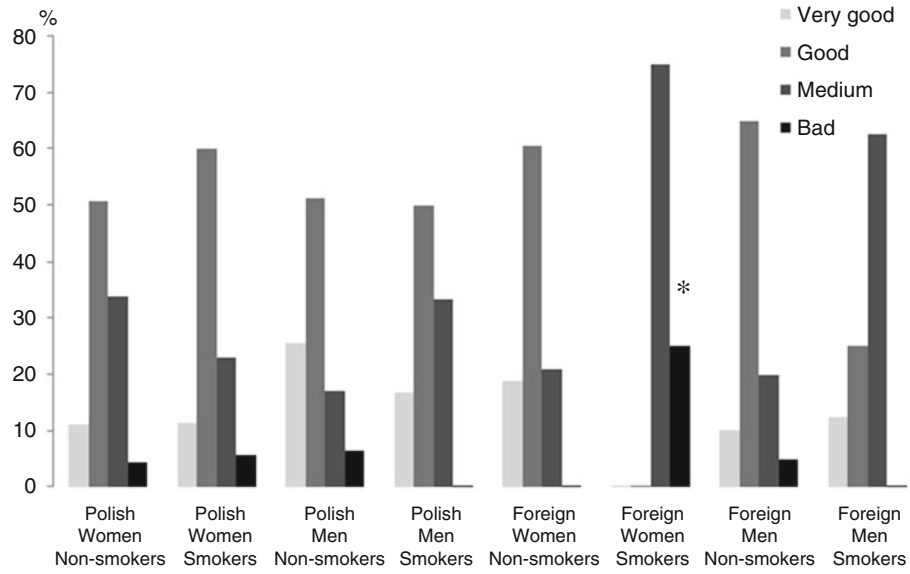

Fig. 2 Self-assessment of physical activity (*p < 0.01 denotes the perception of physical fitness by foreign female students as bad significantly more often compared with the students of all the other groups)

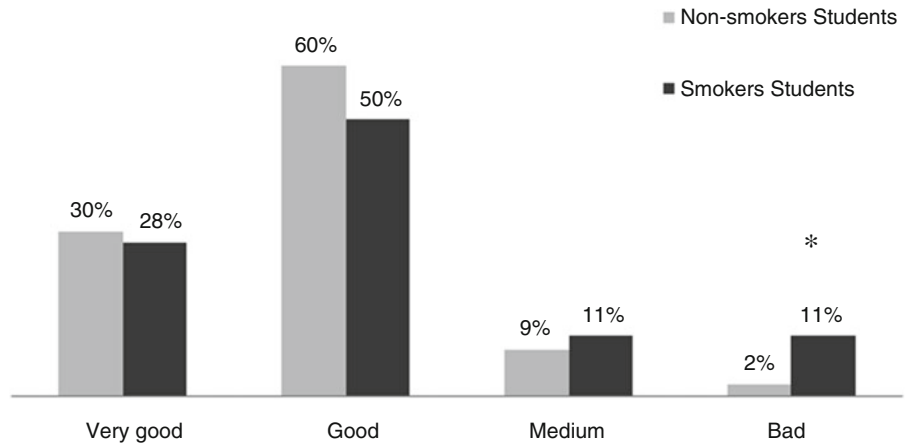

Fig. 3 Self-assessment of health (*p < 0.01 denotes a significant difference in the perception of health status as bad between smoking and non-smoking students)

experiencing mild or moderate pain. Smokers, in particular the foreign female smokers, used painkillers more frequently (Pearson's Chi^2: 27.8, df = 9, p = 0.001) (Table 1). There were no differences among the groups of male subjects, many of whom declared that they did not use painkillers at all.

When asked about satisfaction with life, most Polish and foreign respondents answered that they were satisfied with life and achievements. However, among the Polish male students a significant difference was observed between smokers and non-smokers, with a disadvantage concerning satisfaction with life in the latter group (Pearson's Chi^2: 13.3, df = 4, p = 0.010) (Table 2). Moreover, most of the respondents also were satisfied with their physical appearance (Table 3).

Table 1 Intake of painkillers by students

Group	1/week	2/week	3/week	>3/week
Polish women non-smokers	1.5	11.8	4.4	1.5
Polish women smokers	0	17.1	8.6	8.6
Polish men non-smokers	4.2	6.3	2.1	0
Polish men smokers	5.9	0	5.9	5.9
Foreign women non-smokers	8.3	4.2	2.1	33.3
Foreign women smokers	0	0	50.0	50.0
Foreign men non-smokers	15.0	15.0	15.0	0
Foreign men smokers	25.0	12.5	12.5	12.5

Data are the percentage of students per group

Table 2 Self-assessment of life satisfaction

Group	Much satisfied	Satisfied	Little satisfied	Unsatisfied	Much unsatisfied
Polish women non-smokers	9.4	57.3	29.7	2.2	1.5
Polish women smokers	17.1	68.6	11.4	0	2.9
Polish men non-smokers	8.3	56.3	27.1	4.2	4.2
Polish men smokers	47.1	29.4	17.7	5.9	0
Foreign women non-smokers	19.2	51.1	27.7	2.1	0
Foreign women smokers	0	25.0	50.0	25.0	0
Foreign men non-smokers	20.0	25.0	30.0	15.0	10.0
Foreign men smokers	12.5	50.0	0	37.5	0

Data are the percentage of students per group

Table 3 Self-assessment of physical appearance

Group	Very good	Good	Rather good	Bad	Very bad
Polish women non-smokers	8.5	51.9	34.1	4.7	0.8
Polish women smokers	6.7	63.3	30.0	0	0
Polish men non-smokers	11.6	55.8	25.6	4.7	2.3
Polish men smokers	11.8	58.8	23.5	59	0
Foreign women non-smokers	10.9	52.2	37.0	0	0
Foreign women smokers	0	75.0	25.0	0	0
Foreign men non-smokers	20.0	55.0	20.0	5.0	0
Foreign men smokers	37.5	37.5	25.0	0	0

Data are the percentage of students per group

4 Discussion

Adamek et al. (2008) have assessed the prevalence of cigarette smoking among the students of a private university in the city of Kutno (geodesy, nursing, and pedagogic faculties) and the awareness of its consequences for health. Cigarette smoking was declared by 39 % of students. Of these 81.9 % were regular smokers, and 18.1 % occasional smokers. Similar findings were made in the present study concerning medical students (60 % of students declared themselves to be non-smokers, 20 % were occasional smokers, and 20 % were regular smokers). However, higher rates of smoking students (31 %) were reported from the University of Gaza, Palestine, and additionally 36 % of those students declared smoking waterpipes (Abu Shomar et al. 2014). Smokers of waterpipes are less aware of the health consequences of smoking than

non-smokers (Sharma et al. 2014). Although the awareness of the existing anti-smoking legislations is generally low, it is higher in smokers than in non-smokers. An increase in the number of smokers among medical students, who should promote health-oriented attitudes in their environment, is a disturbing trend (Akl et al. 2011; Sutfin et al. 2012).

The self-assessment of health should be an integral element of general health assessment. Therefore, we addressed this question to students in the present survey. In research carried out by Adamek et al. (2008), the largest proportion of the surveyed group assessed their health as good (56 %) or very good (42 %). In the present study, about 60 % of respondents assessed their health as good, but significantly fewer medical students (about 22 %) assessed it as very good. We found no significant differences in the self-assessment of health between Polish and foreign students (mainly from Scandinavian countries, i.e., Norway and Sweden). This is an important observation, because a very positive self-assessment of health, both in male and female subjects, has been reported in studies carried out by Kasmel et al. (2004) in Finland. The self-assessment of health there was more positive than those concerning either gender in other Baltic countries. In Finland, self-assessment of health is closely associated with health-oriented behaviors, but in other Baltic counties the associations are weak or unstable. The results suggest that in Finland the self-assessed health status is related to health-oriented behaviors and can be used as a relevant measure. In another Baltic countries (Estonia, Latvia, Lithuania), factors other than health-oriented behaviors may be more relevant to the self-assessment of health status (Kasmel et al. 2004). Similar disproportions in self-assessment have been observed among secondary school adolescents in different European countries (Wojnarowska et al. 2008). Scores from the self-assessment of health were best among adolescents from Greece and Slovakia, and poorest among those from Latvia and Malta. However, during the same research period in Poland, 47 % of male and 37 % of female subjects assessed their health as very good.

A prospective study of middle-aged British men has revealed that the self-assessment of health status was strongly associated with mortality. Men who reported poor health had an eight-fold increase in total mortality compared with those reporting excellent health. Those who assessed their health as poor were manual workers, cigarette smokers, and often heavy drinkers. Half of those with poor health suffered from chest pain on exertion and other chronic diseases. Thus, self-assessment of health status appears to be a good measure of current physical health and risk of death, which could be useful in both clinical and epidemiological situations (Wannamethee and Shaper 1991). In the present study we found no significant differences in the incidence of chronic diseases and pain between the analyzed groups. Most respondents did not yet experienced the consequences of cigarette smoking. That might has to do with a relatively short smoking period and a high vitality of the young human body. Interestingly, we found a significantly higher intake of painkillers in smoking female students, although they did not report frequent pain (headache, abdominal pain, or back pain). There seems to be a biological plausibility that smokers may have a greater sensitivity to pain. Smokers also may more frequently experience agitation and emotional instability characteristic of adolescence, accompanied by somatic or hormonal disorders. These factors may have a bearing on a greater use of painkillers by young smokers, but has not yet been explored in research. A more frequent intake of painkillers by females than by their male peers is in harmony with the findings of others (Hoag 2008). This also may result from physiological and also cultural factors. Social acceptance of speaking openly about personal medical problems and emotional states is different for men and women, but also differs depending on the local culture and tradition (Wojnarowska et al. 2008).

Since adolescents are particularly focused on their physical appearance and are frequently dissatisfied with their body image, which often clearly lowers self-esteem, we asked relevant questions to the respondents in the present survey. Most students were satisfied with their

appearance. However, another study on younger adolescents from a secondary school revealed that a significantly higher number of boys than girls are more satisfied with their appearance. We did not find any clear inter-gender differences regarding university students. Satisfaction with personal appearance is a positive trend, as dissatisfaction can reduce self-esteem in young people and prompt them towards cosmetic interventions which may sometimes have negative health consequences, especially when mass media promote a specific type of perfect body. A relatively high prevalence of smoking among medical students, their low level of physical activity, and the poor awareness of the consequences of smoking revealed in the present study are disturbing, because the knowledge on that is crucial for doctors, who are supposed to promote a healthy lifestyle (Sutfin et al. 2012; Arnett et al. 2012; Miller et al. 2011).

5 Conclusions

1. The presence of negative health behaviors and poor physical activity was observed among medical students.
2. Students did not see a negative influence of cigarette smoking on their health, well-being, or physical appearance, most likely because their tobacco abuse was not long-term enough to reveal the perceivable effects of smoking cigarettes on the body.
3. Young women addicted to the habit of smoking take more painkillers, despite no apparent ill health problems.
4. A promotional campaign should be introduced particularly at medical universities since medical students should support healthy behavioral patterns with their own attitudes.

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

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The Role of Inspiratory Muscle Training in the Process of Rehabilitation of Patients with Chronic Obstructive Pulmonary Disease

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Abstract

Chronic obstructive pulmonary disease (COPD) adversely affects the quality of life and life expectancy of patients. Shortness of breath, cough, and fatigue in lower limbs are the main reasons limiting physical activities of patients. The lack of physical activity results in poorer muscle strength. The latest guidelines regarding breathing rehabilitation in COPD patients emphasize a significant role of inspiratory muscle exercises. The objective of the present study was to evaluate the effects of an 8-week long inspiratory muscle training, interval training on a cycle ergometer, and training combining both kinds of rehabilitation, on pulmonary function, health-related quality of life, and the tolerance to exercise in patients with COPD. The study was conducted in a group of 43 patients with diagnosed COPD stage II and III according to GOLD. They were randomly divided into four training groups: inspiratory muscle training (Group 1), cycle ergometer training (Group 2), cycle ergometer and inspiratory muscle training (Group 3), control group - patients who did not participate in any rehabilitation programs (Group 4 - control). Before the rehabilitation process and after its completion the patients were medically examined, they completed a health-related quality of life questionnaire, performed a 6-min walk test, spirometry, and a treadmill exercise test according to the modified Bruce protocol. The results demonstrate a significant improvement in the quality of life measured for Group 3 in comparison with the control group.

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Keywords
COPD • Inspiratory muscles • Lung ventilation • Quality of life • Physical training • Pulmonary rehabilitation

1 Introduction

Chronic obstructive pulmonary disease (COPD) adversely affects the health-related quality of life and life expectancy of patients. The major symptoms of COPD such as shortness of breath, cough, and fatigue in lower limbs result in the lack of exercise tolerance. Poor physical strength results in increased breathing effort despite little physical activity. Low physical fitness in COPD patients is associated with higher mortality (Oga et al. 2003).

The 2013 ATS/ERS guidelines emphasize the role of inspiratory muscle exercise in the rehabilitation programs, particularly of importance in patients with decreased strength of these muscles (Spruit et al. 2013). In addition, studies regarding physical rehabilitation in COPD patients demonstrate that training of lower limbs increases exercise tolerance (Reis et al. 1995). In particular, interval training of lower limbs seems to offer the greatest improvement in exercise tolerance and quality of life. Furthermore, interval training is best tolerated by this group of patients (Vogiatzis et al. 2004, 2005). Therefore, in the present study we set out to determine the effects of an 8-weeklong inspiratory muscle training, interval leg training on a cycle ergometer, and training combining both kinds of rehabilitation, on pulmonary function, health-related quality of life, and the tolerance to exercise in patients with COPD.

2 Methods

The study comported with the guideline of the Declaration of Helsinki for Human Experimentation and was approved by a local Ethics Committee. Forty three patients diagnosed with COPD were enrolled into the study. The inclusion criteria were the following:

- COPD treated for at least one year, stage II and III according to GOLD (2014);
- 50–70 years old;
- stable clinical condition with no exacerbations over the period of 4 weeks before the study,

The presence of any of the following excluded patients from the study:

- participation in pulmonary rehabilitation in the year preceding the study;
- diagnosed bronchial asthma;
- long-term home oxygen therapy;
- clinically significant diseases of the cardiovascular system;
- any uncontrolled chronic disease;
- muscle and nervous disorders reducing the patient's mobility;
- mental disorders preventing contact and cooperation with the patient.

The COPD patients were randomly divided into 4 groups:

- Group 1: inspiratory muscle training (IMT);
- Group 2: cycle ergometer training (CET);
- Group 3: IMT plus CET;
- Group 4: control group; patients did not participate in any rehabilitation programs.

All the patients continued their pharmacological treatment during the study period. Demographic characteristics of the patients studied are shown in Table 1.

In order to qualify the patient to the rehabilitation program and to evaluate its end effects, the

Table 1 Basic characteristics of the patient groups

	Group 1 IMT (*n* = 8)	Group 2 CET (*n* = 9)	Group 3 CET + IMT (*n* = 13)	Group 4 Control (*n* = 13)	p
Women	6 (75.0 %)	3 (33.3 %)	3 (23.1 %)	6 (46.2 %)	0.12[b]
Men	2 (25.0 %)	6 (66.7 %)	10 (76.9 %)	7 (53.8 %)	
Age (years)	63.4 ± 9.8	62.3 ± 5.2	61.5 ± 6.1	65.5 ± 7.0	0.52[a]
BMI (kg/m^2)	26.1 ± 5.9	28.2 ± 6.2	28.8 ± 6.2	27.8 ± 4.9	0.77[a]
Pack/years	25.8 ± 13.2	40.7 ± 19.4	33.9 ± 15.4	37.7 ± 23.8	0.40[a]

Quantitative data are means ± SD
Pack/years = the number of packs of cigarettes smoked per day times the number of years the person has smoked
[a]one-way ANOVA
[b]Chi^2 test

following tests were employed before and after the program completion:

- St. George's Respiratory Questionnaire (SGRQ),
- pulmonary function using a MasterScreen Pneumo spirometer (Jaeger-CareFusion, San Diego, CA);
- maximal inspiratory pressure measurement (PI_{max}), taken as a measure of the inspiratory muscle strength;
- electrocardiographic treadmill exercise test according to the modified Bruce protocol (Trackmaster TMX 425, Oxford, UK). The intensity of the test was limited to submaximal values and the result were evaluated in metabolic equivalents (MET).

The patients in Groups 2 and 3 were subjected to the interval training on a cycle ergometer. The training took place over 8 weeks, 3 times a week in an ambulatory setting under the supervision of a cardiologist. Each training session began (warm-up) and finished (relaxation) with a pedaling at a load of 10 W for 3 min. The training load was calculated individually for each patient on the basis of the exercise test results. The duration of a training session was initially 23 min and then it was gradually increased up to 45 min as shown in Table 2.

The patients in Groups 1 and 3 were subjected to the inspiratory muscle training performed by themselves at home on a Threshold IMT (Respironics; Philips Healthcare, DA Best, The Netherlands). The training took place over 8 weeks, 5 times a week, twice a day for 5–15 min (depending on the stage of the training program) in the morning and evening at fixed times. The level of an individual training load was set on the basis of PI_{max} value (Table 3).

Data were given as means ± SD. Statistical comparisons for inter-group differences were made with one-way ANOVA or the Kruskal-Wallis test. Wilcoxon test was used for paired samples. Categorical data were compared with a Chi-squared test. $P < 0.05$ was set as the definer of statistical significance of differences.

3 Results

The training methods were well tolerated by the patients, with no detectable side effects, in the three training groups. A trend for growth in exercise tolerance was observed in Groups 2 and 3. A significant improvement was observed in the total SGRQ score in Group 3 (CET + IMT) in comparison with the control group; detailed results are shown in Table 4.

4 Discussion

Pulmonary rehabilitation is used to reduce symptoms of COPD and improve quality of live. Most of clinical trials focused rehabilitation on lower extremities training alone or combined with respiratory muscle training. Larson et al. (1999) have shown reductions in the perception of the feeling of dyspnea and lower limb

Table 2 Schedule of cycle ergometer training (CET)

Week I	Weeks II & III	Week IV	Weeks V & VI	Weeks VII & VIII
23 min	28 min	34 min	40 min	45 min

Table 3 Schedule of inspiratory muscle training (IMT)

Week of training	I	II	III	IV	V	VI	VII	VIII
Duration of training session (min)	2 × 5	2 × 8	2 × 11	2 × 11	2 × 13	2 × 13	2 × 15	2 × 15
Training load (% PI_{max})	30	40	40	50	50	60	60	60

Table 4 Results of pulmonary rehabilitation in the COPD patient groups

	Group 1	Group 2	Group 3	Group 4	p
Initial MET	5.8 ± 2.6	4.4 ± 1.5	6.5 ± 3.0	5.4 ± 2.2	0.26[a]
Final MET	5.2 ± 1.7	5.1 ± 1.5	6.9 ± 3.1	5.2 ± 2.0	0.15[a]
Initial vs. Final	$p = 0.34^b$	$p = 0.32^b$	$p = 0.51^b$	$p = 0.14^b$	×
Initial FEV1 (L)	1.1 ± 0.4	1.3 ± 0.4	1.8 ± 0.7	1.8 ± 0.6	**0.01[a]**
Final FEV1 (L)	1.1 ± 0.5	1.3 ± 0.3	1.8 ± 0.7	1.7 ± 0.5	**0.03[a]**
Initial vs. Final	$p = 0.77^b$	$p = 0.45^b$	$p = 0.81^b$	$p = 0.14^b$	×
Initial FEV1 (% predicted)	49.9 ± 17.2	49.0 ± 17.1	62.4 ± 18.5	68.4 ± 20.0	**0.05**[a]
Final FEV1 (% predicted)	50.1 ± 20.4	51.1 ± 14.6	61.9 ± 21.0	65.3 ± 19.2	0.194[a]
Initial vs. Final	$p = 0.94^b$	$p = 0.53^b$	$p = 0.89^b$	$p = 0.22^b$	×
Initial SGRQ total score	47.5 ± 16.9	52.4 ± 15.5	57.7 ± 19.0	47.2 ± 16.0	0.40[a]
Final SGRQ total score	47.2 ± 16.0	50.6 ± 12.6	48.2 ± 17.1	47.5 ± 19.4	0.97[a]
Initial vs. Final	$p = 0.92^b$	$p = 0.56^b$	$\mathbf{p = 0.02}^b$	$p = 0.87^b$	×

Data are means ± SD

MET metabolic equivalents, *FEV1* forced expiratory volume in one second, *SGRQ* St. George's Respiratory Questionnaire

[a]Kruskal-Wallis test

[b]Wilcoxon test; significant changes were bolded out

fatigue in a group of COPD patients subjected to a 4-month clinical trial with home-based cycle ergometry and inspiratory muscle training. Another clinical trial involving COPD patients, with home-based inspiratory muscle training for 1 year, has demonstrated an increase in inspiratory muscle strength and improvement of health-related quality of life (Beckerman et al. 2005). The present study showed that cycle ergometer training performed in a hospital setting for 8 weeks, accompanied by an additional home-based inspiratory muscle training, significantly improved health-related quality of live. We conclude that a combination of hospital and following home-based methods of pulmonary rehabilitation may benefit quality of life in COPD patients after a relatively shorter time compared with other relevant methods, which makes it an attractive alternative.

5 Conclusions

The highest effectiveness of rehabilitation demonstrate the group with combined both types of training.

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

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Socioeconomic Effects of Chronic Obstructive Pulmonary Disease from the Public Payer's Perspective in Poland

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Abstract

Chronic obstructive pulmonary disease (COPD) is currently the third most common cause of death worldwide and the total number of people affected reaches over 200 million. It is estimated that approximately 50 % of persons having COPD are not aware of it. In the EU, it is estimated that the total annual costs of COPD exceed €140 billion, and the expected increase in the number of cases and deaths due to COPD would further enhance economic and social costs of the disease. In this article we present the results of cost analysis of health care benefits associated with the treatment of COPD and with the disease-related incapacity for work. The analysis is based on the data of the National Health Fund and the Social Insurance Institutions, public payers of health benefits in Poland. The annual 2012 expenditures incurred for COPD treatment was €40 million, and the benefits associated with incapacity

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for work reached more than €55 million. The extent of these expenditures indicates that it is necessary to optimize the functioning system, including the allocation of resources for prevention, social awareness, and detection of COPD at early stages when treatment costs are relatively low.

Keywords
Chronic obstructive pulmonary disease • Health care benefits • Prevention • Social awareness • Treatment costs

1 Introduction

It is estimated that globally 3.1 million people die each year due to chronic obstructive pulmonary disease (COPD). According to the World Health Organization (WHO 2014), the disease was the third leading cause of death worldwide in 2012. The exact incidence of COPD is unknown, but it likely is very high as the estimate is that the number of symptomatic patients, i.e., those undergoing treatment, does not exceed 50 % of those being sick; the situation similar to what there is in bronchial asthma. According to the estimates, moderate-to-severe COPD affected around 64 million people in 2013 (WHO 2013a) and the total number of persons suffering from COPD has reached 210 million (Fletcher et al. 2011; Mannino and Braman 2007). Asthma, in turn, is present in 235 million people (WHO 2013b), although according to the Global Initiative for Asthma, this number may actually exceed 300 million (Masoli et al. 2004).

The prevalence of COPD in Europe is high. Approximately 5–10 % of adults over 40 suffer from this disease, with a higher incidence in men than women. According to other estimates, the disease affects 8–10 % of the population aged 45 and over (Ko and Hui 2012; Afonso et al. 2011). The prevalence of COPD in those over 40 years of age, confirmed by spirometry, is 8.9 % (Halbert et al. 2006). In studies conducted in Poland, the prevalence of COPD ranged from 10 % (Niepsuj et al. 2002) to 26 % (Nizankowska-Mogilnicka et al. 2007) in persons over 40 years of age.

In the EU countries, the disease affects about 23 million people. There has been a marked increase in COPD occurrence with age. In people older than 70, the prevalence is 20 % in men and 15 % in women. However, the disease might be overdiagnosed in this age group due to the adoption of the Tiffeneau index threshold, which decreases physiologically in the elderly. The most important single etiological factor for COPD is smoking. The prevalence of the disease is closely related to the epidemic smoking, with a delay of 20–30 years. Approximately 40–50 % of lifelong smokers will go on to develop COPD, compared with just 10 % never-smokers. The research presented in the European Lung White Book (ERS 2013) indicates that approximately 15–20 % of cases of COPD are caused by exposure to air pollution in the workplace and living environment. Taking into account high concentrations of air pollutants in Poland, especially particulate matter and benzo(a)pyrene, in comparison with most other EU countries, the issue of the obstructive diseases like COPD and asthma could be of great importance. The risk of developing COPD in Europe is also associated with low socio-economic and education status.

COPD is always accompanied by impaired respiratory function in the form of obstruction, hyperinflation, and emphysema. It is estimated that about 50 % of people live with the disease not being aware of it, although the latest literature information indicates that this percentage may be as high as 60–85 % (Decramer et al. 2012; Dąbrowiecki et al. 2013). Lack of awareness of the disease most often results from the absence of symptoms or their

misinterpretation. It is often also the question of lack of a proper medical examination or a pulmonary function test. The European Lung White Book shows that about one million Europeans, inclusive of 700,000 EU citizens, died due to lung disease in 2008. Lung disease was the third leading cause of death after ischemic heart disease and cerebrovascular diseases. Further, lung cancer and COPD-related mortality is expected to rise in the coming decades. About 5.2 million disability-adjusted life years (DALY) is reported yearly as a result of lung diseases. In the EU, total costs of lung diseases amounted to more than €379.6 billion and those of COPD alone stood at €141.4 billion in 2011. The latter cost consisted of direct costs of health care – 23.3 billion (16 %), indirect costs of lost productivity – 25.1 billion (18 %), costs of 1.7 million DALY in COPD – €93 billion (56 %). As part of direct costs, the costs of medications were €7.1 billion (30 %), outpatient care – €8.9 billion (38 %), and hospitalization – €7.3 billion (32 %).

In Poland, no representative nationwide epidemiological studies concerning COPD have been conducted, whereas partial studies have shown the presence of the disease in 10 % of the population aged over 40; with the exception of one study pointing to the prevalence of 26 % (Śliwiński et al. 2014). The estimation made for the capital city of Warsaw demonstrates the annual loss of 1600 DALY due only to PM_{10} and NO_x emissions related to road transport (Adamkiewicz et al. 2015). According to the estimates of Bednarek et al. (2008), the disease is at mild-to-moderate stage in approximately 80 % of cases.

Too late diagnosis and inappropriate treatment contribute to the progression of the disease and exacerbations, often requiring hospitalization. Exacerbations are a poor prognostic factor, they negatively influence the quality of life, respiratory efficiency, and limitation physical activity, which increases the risk of occurrence of other diseases, e.g., thromboembolism (Niesinka et al. 2007; Tillie-Leblond et al. 2006). The prognosis worsens with each significant exacerbation. An almost 40 % mortality rate has been observed during one year after exacerbation requiring hospitalization, and mortality exceeds 50 % in patients requiring intensive care (Yohannes et al. 2005; Seneff et al. 1995). The advanced stage of COPD is related to emotional and social problems. It is estimated that 25 % of patients with COPD experience anxiety or depression; among 90 % of these patients both problems coexist, which increases the risk of COPD exacerbations, worsens quality of life, shortens the survival time, and increases treatment costs (Kunik et al. 2005).

The problem of a relatively small percentage of people who are aware of suffering from COPD indicates the importance of an early identification of the disease and taking up treatment to slow down its progression, to maintain the quality of life of people at an acceptable level, and to ensure a rationalized health care financing. The present article presents the analysis of annual expenses incurred by the institutional Polish national payer concerning the health care benefits associated with COPD treatment COPD and benefits resulting from work absenteeism.

2 Methods

This study was approved by an institutional review board for research. The analysis of COPD-related expenditures was based on the data from the Polish National Health Fund – a state institution funding health care benefits for insured persons and reimbursing medicines with funds collected from mandatory health insurance contributions, and from the Social Insurance Institution – a state public law institution fulfilling social security function.

The data from the National Health Fund concerned expenditures related to the implementation of contracts in terms of health care benefits associated with the treatment of COPD in 2012, including hospital treatment, outpatient specialty care, primary health care, and to a small extent benefits for medical rehabilitation and preventive health programs. The health care benefits provided were stratified by sex, age, and region of residence. The data provided by the Social

Insurance Institution concerned the extent of sickness-related absenteeism and medical certification, and the amounts of benefits paid out accordingly (National Health Fund 2013).

The aim of the analysis was to identify the possible directions of rationalization of medical care, leading to a reduction of social, medical, and economic consequences of COPD in Poland. Based on the National Health Fund (2013) data, analysis of treatment financing of COPD patients in 2012 was carried out. The stratification into two age-groups: 0–65 and 65+ years of age, done during the analysis of financing of health services to patients results from the retirement age threshold adopted by the state in the period of research, while the 16 regions of residence (voivodeships) correspond to the administrative division of the country. Specifications were prepared by individual voivodeships. Based on the data from the Social Insurance Institution, a statistical analysis of sickness absenteeism and disability pension benefits was conducted in the case of insured persons diagnosed with COPD. These data allowed for a quantitative analysis of sickness-related incapacity for work to the accuracy of a single disease classification unit, defined according to the International Statistical Classification of Diseases and Related Health Problems ICD-10.

3 Results

3.1 National Health Fund Data Concerning the Financing of COPD Treatment Benefits

In 2012, the National Health Fund in Poland disbursed benefits associated with COPD hospital treatment, outpatient care, and medical rehabilitation in the approximate amount of €39.3 million. Three fourth of these costs were expenses related to hospital treatment (29.8 million), while the costs of outpatient specialty care accounted for 8.1 million (20.7 %), with medical rehabilitation running third at €1.4 million (3.5 %) (National Health Fund 2013).

Across-country quantitative analysis of the costs associated with the three types of benefits outlined above showed that €25.3 million (64.5 %) was incurred by the age-group 65+ years, while €14 million (35.5 %) by the age-group 0–65 years. Translating these figures into the number of benefits provided, out of the total of 706,345 of benefits settled in the country, the majority (56.0 %) concerned the persons 65+ years of age, of which 90.6 % accounted for provisions provided by outpatient specialty care, 8.9 % for hospital treatment, and 0.6 % for medical rehabilitation. For comparison, the National Health Fund financed medical advice concerning the primary health care amount to €1.42 million.

Summing up, the provisions financed from the budget of the National Health Fund in 2012 amounted to €40.7 million and the health care covered 513,878 patients diagnosed with COPD. The percentage of patients aged 0–65 was to 45.8 %, with the remaining fraction aged >65 (National Health Fund 2013). There were differences among voivodeships in terms of the total monetary value and the number of financed health care benefits. A 5-fold difference existed between voivodeships with the lowest (Lubosz Voivodeship in western Poland, respectively) and highest (Masovian Voivodeship in central Poland) value of settled benefits (Fig. 1), and nearly a 7-fold difference in the number of benefits (the lowest in Opole Voivodeship and the highest in the southern Silesian and central Masovian Voivodeships) (Fig. 2). These disparities concerned both inpatient and outpatient specialist care. At the same time, as expected, a very clear conformity of results in the different voivodeships was achieved, comparing the values and the numbers of funded provisions of various kinds (Figs. 3, 4, 5, and 6). The analysis at regional levels confirmed the national structure of expenditures to finance benefits as above outlined in conjunction with the population age.

In all voivodeships, a more than two-fold difference in the value of benefits funded for hospital treatment was indicated in the age groups studied, wherein the lowest values were

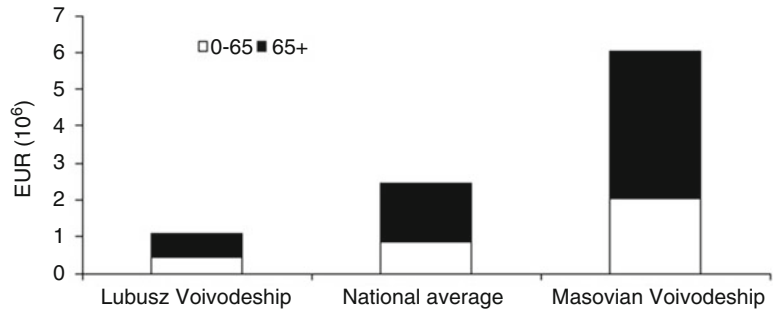

Fig. 1 Comparison of regional lowest and highest monetary value of settled health care benefits with the national average, stratified by age-groups

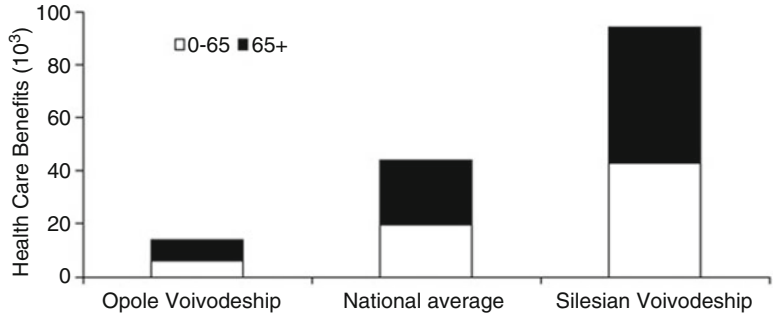

Fig. 2 Comparison of regional lowest and highest number of settled health care benefits with the national average, stratified by age-groups

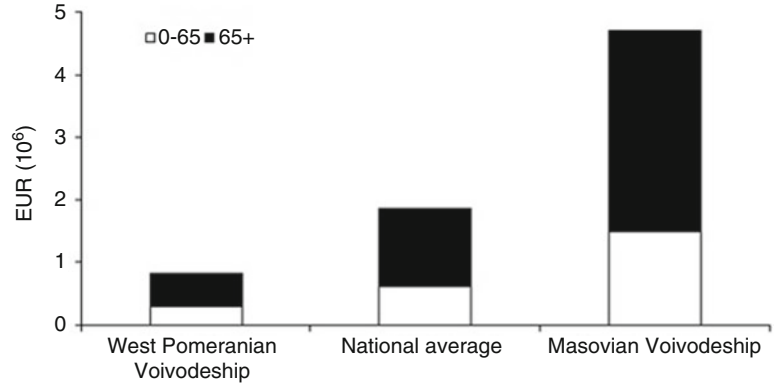

Fig. 3 Comparison of regional lowest and highest monetary value of settled benefits regarding hospital treatment with the national average, stratified by age-groups

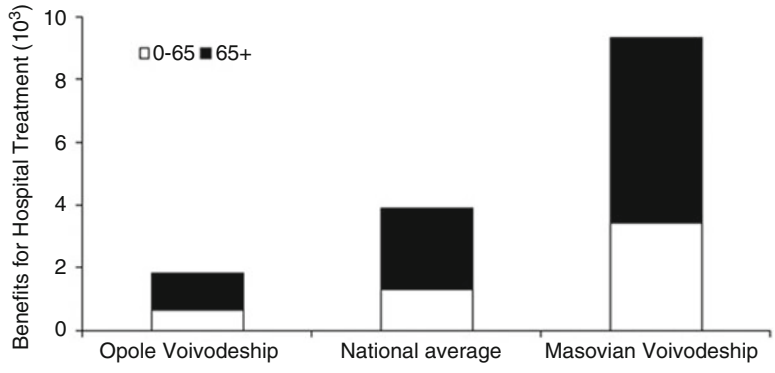

Fig. 4 Comparison of regional lowest and highest number of settled benefits regarding hospital treatment with the national average, stratified by age-groups

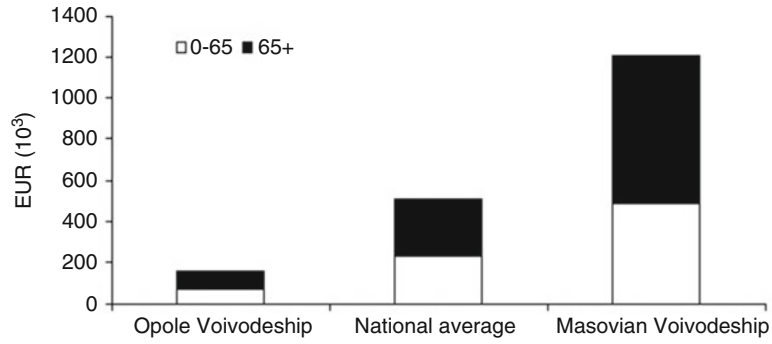

Fig. 5 Comparison of regional lowest and highest monetary value of settled benefits regarding outpatient specialist care with the national average, stratified by age-groups

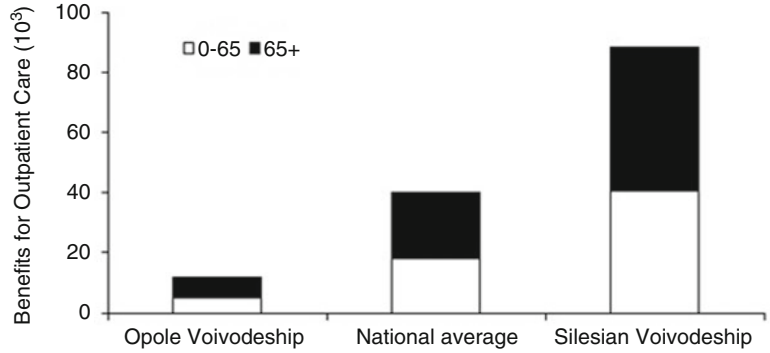

Fig. 6 Comparison of regional lowest and highest number of settled benefits regarding outpatient specialist care with the national average, stratified by age-groups

indicated in West Pomeranian (north-western part of the country) and Opole Voivodeships, and the highest in Masovian Voivodeship (Fig. 3). There were 32.6 % of benefits in the amount of €9.7 million financed in the 0–65 age group and 67.4 % of benefits in the amount of €20.1 million in the 65+ age group. This trend was also reflected in the number of settled benefits related to hospital treatment, with the lowest number indicated in Opole Voivodeship and highest in Masovian Voivodeship (Fig. 4). There were 21,136 cases of hospitalization reported in the 0–65 age group and 41,398 cases of hospitalization in the group 65+ years of age. Both nationally and in individual voivodeships, twice as many benefits were settled in relation to hospital treatment in the 65+ than 0–65 age group. At the same time, a clear conformity was demonstrated at the regional level regarding the value and number of financed provisions. This conformity was maintained also when broken down into the two age groups.

As regards the remaining two types of benefits, outpatient care and medical rehabilitation, the age structure was akin. Among 639,824 of outpatient specialist services, 45.2 % of the costs incurred (€3.7 million) concerned the 0–65 age group and 54.8 % (€4.5 million) the 65+ group. A correlation between the value and the number of settled benefits at the voivodeship level was demonstrated. The lowest value and number of settled benefits were demonstrated in Opole Voivodeship and the highest in Masovian and Silesian Voivodeships (Figs. 5 and 6). This correlation also was present in individual age groups.

In case of medical rehabilitation, 41.2 % of the costs concerned the 0–65 age group (€0.6 million), whereas 58.8 % concerned the 65+ age group (€0.8 million). In 2012, the National Health Fund (2013) settled 742,082 cases of medical advice provided to patients with COPD in the framework of primary health care, of which 58.8 % was given to persons 65+ years

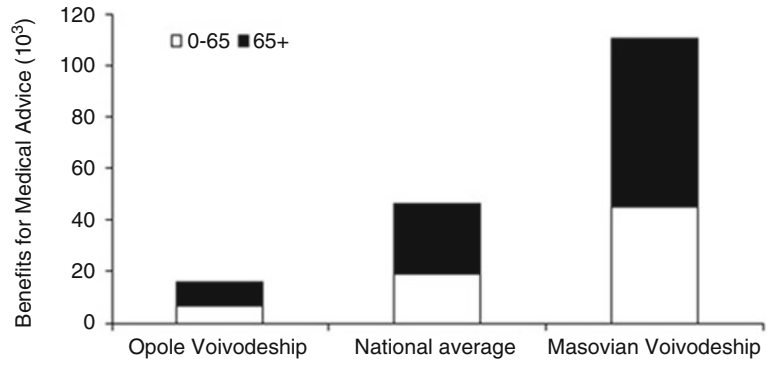

Fig. 7 Comparison of regional lowest and highest number of settled cases of medical advice provided within primary health with the national average, stratified by age-groups

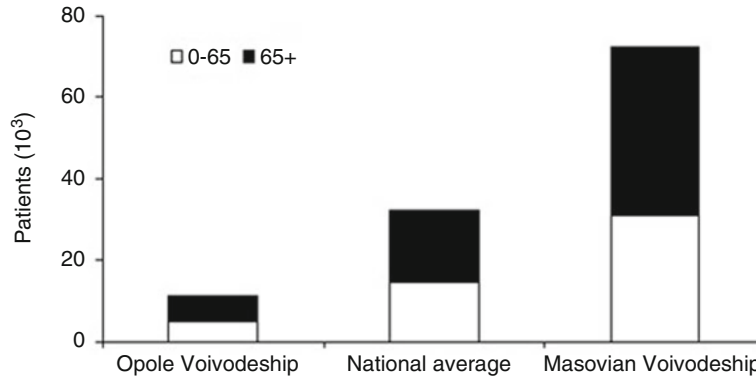

Fig. 8 Comparison of regional lowest and highest number of patients with COPD with the national average, stratified by age groups

of age and 41.2 % to those aged 0–65. Quantitative relations between individual voivodeships of the country were similar to those shown in Figs. 5 and 6 (Fig. 7).

Costs related to the prevention of tobacco-related diseases constitute a relatively small part of the expenses incurred in connection with the incidence of COPD in Poland. In 2012, the National Health Fund settled in this regard a total of 25,008 benefits in all 16 voivodeships, where benefits, for which a major disease classification unit was COPD, accounted for 0.8 % of this figure, i.e., 194 benefits across the country. In that year, the National Health Fund (2013) reported 513,878 patients with COPD nationwide, with the lowest and highest voivodeship incidence similar to those described for the outpatient specialist care and medical rehabilitation above shown (Fig. 8).

The percentage of patients in the general population with the primary diagnosis of COPD was similar in individual voivodeships. It amounted to 1.3 % nationwide, and ranged from 1.1 in Opole Voivodeship to 1.6 % in Swietokrzyskie Voivodeship in southern Poland. Given the estimated number of COPD people affected in Poland of about two million, which represents 5.2 % of the population, the results show that the disease remains grossly undiagnosed.

3.2 Social Insurance Institution Data on Funding Benefits due to Sickness-Related Absenteeism Resulting from COPD

In 2012, the Social Insurance Institution registered about 26,000 medical certificates of temporary incapacity for work due to COPD-related illness. This represents 0.2 % of all medical certificates issued in 2012 regarding

temporary incapacity for work due to personal illness. A total number of days of sickness absenteeism due to COPD was 348,600 nationwide, while the average length of a sick leave was 13.4 days, which was higher than the average for all diseases combined, amounting to 12.5 days). The average accumulated COPD-related absenteeism, a sum of individual's sick leaves in the year, amounted to 28.1 days. The number of persons insured by the Social Insurance Institution, for whom at least once during the year a medical leave due to COPD was issued amounted to 12,400, representing 0.22 % of all persons to whom a sick-leave certificate was issued due to any reason. At the voivodeship level, regions with the highest and lowest numbers of sick-leave certificates corresponded to the voivodeships in which the most and fewest medical provisions were settled, respectively. That year, preliminary statements entitling to the rehabilitation benefit in relation to incapacity for work resulting directly from COPD were issued to 288 people. This represented 0.35 % of all initial medical statements. Subsequent statements were issued to 283 patients, representing 0.42 % of all subsequent statements. Preliminary medical statements on incapacity for work due to COPD, forming the basis for granting a relevant disability pension were issued to 488 patients, which is 1.10 % of all preliminary statements issued in 2012. As in case of sick-leave certificates, the highest number of statements for the purposes of disability pension was reported in voivodeships with the greatest value and number of settled medical benefits. Subsequent statements for disability pension purposes were issued to 3844 patients with COPD, which accounted for 1.44 % of all subsequent statements issued by the Social Insurance Institution for disability pensions.

Under the program of disability pension prevention, rehabilitation covered 859 people targeted due to COPD in 2012. This represented 1.21 % of all persons covered by the program. The expenditure incurred by the Social Insurance Institution with regard to benefits associated with incapacity for work due to COPD amounted to €56.4 million that year. This amount represented 0.78 % of the total expenditure incurred by the Social Insurance Institution as regards incapacity for work caused by various diseases. The breakdown of the €56.4 million was as follows: 89.3 % were incapacity for work pensions, 7.9 % were benefits paid in connection with sickness absenteeism, 1.9 % were rehabilitation benefits, 0.48 % were expenses incurred in connection with medical rehabilitation, and 0.4 % were social assistance benefits. Benefits due to incapacity for work caused by COPD predominated in the above expenditures, being received by approximately 19,100 beneficiaries in a total amount of €50.35 million. At the voivodeship level, as expected, the lowest number of disability pension beneficiaries was in the regions where there were the fewest persons who received COPD-related medical benefits such as Opole Voivodeship (Fig. 9).

The costs generated by patients with COPD are clearly underestimated, which may require a rethink on how to make more true estimates. Perhaps another form of data collection by the Social Insurance Institution, such as entering the

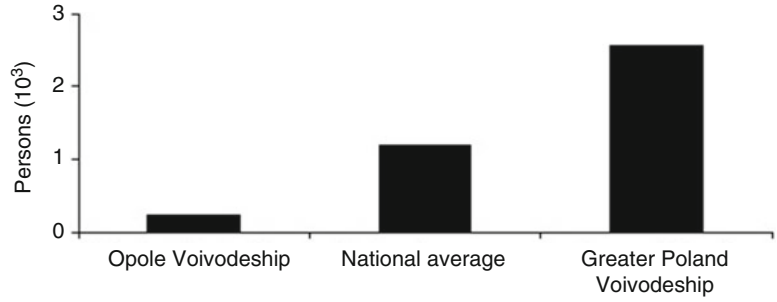

Fig. 9 Comparison of regional lowest and highest number of persons with COPD-related disability pension, stratified by age groups

main and co-occurring diseases, in the documents filled out by doctors would help in more accurate monitoring of this group of patients. COPD is a multi-organ disease. It covers the lungs, but persisting inflammation also affects the cardiovascular system. Heart attacks and strokes are thrice as often in COPD as in the general population and type 2 diabetes develops more often, resulting in hospitalization. Due to the weakness in data collection, the diagnosis of chronic obstructive pulmonary disease with acute lower respiratory infection (coded J44.0 by ICD-10-CM) may appear in a secondary place and thus may not be the essential part of official statistics.

4 Discussion

The finding of this study are that the number of patients with a primary diagnosis of COPD, taking advantage of medical benefits, constitutes only 1.33 % of the total population of Poland. This result is consistent with that obtained during the Polish Spirometry Day in 2011 (Dąbrowiecki et al. 2013) when 1.1 % of respondents declared having had a diagnosed COPD, while pulmonary function tests showed objectively the presence of obstruction in 12.3 % of patients. Likewise, Badyda et al. (2013) have found obstruction in 8.4 % of subjects performing spirometry, while the study participants' declaration amounted to 0.4 % of diagnosed COPD. In both cases, a significant proportion of respondents were not aware of the disease, nor did they inform of it in the survey for some other reasons. In a study conducted in France in 2004 similar values were achieved – the incidence of COPD according to personal declarations in the general population was estimated at 1.3 % (Detournay et al. 2004). These results signal an important social problem which from the point of view of national economy also translates into an economic effect. Health care costs of persons with more advanced COPD grow significantly compared with the costs of treatment of mild stages of the disease.

The present study demonstrates the annual expenditure related to hospital treatment, out-patient specialist care, and medical rehabilitation in the amount of *ca.* €39 million. Approximately 76 % of these costs were expenses related to hospital treatment (€29.8 million), out-patient specialist care constituted about 21 % of these costs (€8.1 million), and medical rehabilitation – less than 4 % (€1.4 million). In the framework of primary health care, the National Health Fund financed medical advice in the amount of €1.42 million. The total value of expenses incurred for medical provisions in 2012 amounted to €40.7 million, which comes to €79 *per* patient *per* year. This shows a profound underestimation of the COPD budget and, possibly, allocating costs to other comorbid conditions such as asthma or ischemic heart disease.

Generally, the costs of treatment of patients with COPD are highly variable and, depending on the country, range from several hundred to several thousand euros *per* year (Hoogendoorn et al. 2014). The Spanish example indicates that with the annual costs at €2000 *per* patient, the largest share (41 %) of costs is taken up by hospitalization, with a smaller share allocated to pharmacotherapy (26 %) and admissions to hospital emergency units (7 %) (de Miguel Diez et al. 2008). In Poland, the 2008 costs of treating a patient with COPD have been estimated as €1000 (Jahnz-Różyk et al. 2011). Treatment of exacerbations in the hospital constitutes 29 % of the total cost, and the cost of medicines and diagnostic tests – 41 % of the total. Population-based studies carried out in southern Germany show that annual costs of treating COPD in one patient amount to little more than €1800, where the cost of medicines is 30 % and the cost of hospitalization – 49 % of the total (Menn et al. 2012). These authors also indicate that in case of a moderate form of COPD the overall costs increase by an average of 54 %. Other estimates suggest, however, that the costs of treatment of moderate stages of COPD may be 3–4-fold higher in comparison with the mild form of the disease, and in the severe form they reach up to 6–10 times the basic cost (Chiang

2008; Jansson et al. 2002; Hilleman et al. 2000). Masa et al. (2004) also have shown that the highest costs are generated by the hospital treatment (41 %), followed by pharmacotherapy (37 %). According to a study carried out in France by Detournay et al. (2004), more than one third of total direct costs were associated with hospitalizations, while 31 % was related to pharmacotherapy.

The relatively high proportion of costs of hospital treatment in Poland, compared with other countries, may result primarily from shifting the costs from specialist and primary care onto hospital treatment, low valuation of specialist medical advice, and a failure to take into account pharmacotherapy in the expenditure structure. The last fact stems from an insufficient amount of reliable data. Since pharmacotherapy costs reach 26–40 % of the total in different countries, such an analysis should be the subject of further research, also in the context of disparities in the number of cases of hospitalization in the age groups studied. Another explanation of the too high share of hospitalization costs *vs*. total costs may be the lack of funding of health education, which translates into a late diagnosis, a more rapid progression to more severe stages, and consequently to treatment of mainly severe disease forms, which is expensive and requires frequent hospitalizations.

A two-fold difference between the 65+ and 0–65 age groups in both the number and value of funded benefits concerning hospital treatment was not reflected in other types of benefits, i.e., outpatient specialist care and medical rehabilitation. This disproportion was present in all 16 analyzed voivodeships, and in some regions the difference exceeds twice the value. It should be emphasized that the limit of 65 years of age had until recently constituted the retirement age in Poland, and thus often the age of a significant deterioration in socioeconomic status of patients. It is pointed out that in Poland a substantial part of old age pensioners and disability pensioners due to the lack of financial reasons do not purchase the medicines prescribed. Therefore, it is possible that a two-fold increase in hospitalizations in the 65+ age group was caused by insufficient pharmacotherapy and, consequently, disease exacerbations. In this context, further studies should be carried out covering this age group to examine whether cases of repeated, multiple, or prolonged hospitalization are due to insufficient pharmacotherapy or are caused by other factors specific for this age group, e.g., late onset of treatment or a large number of smokers in this generation (Soler et al. 2006).

The costs of treating COPD represent a serious economic burden, particularly for health care systems in industrialized countries (Pauwels and Rabe 2004). In 2005, in the US alone, as shown by Foster et al. (2006), annual direct costs of COPD reach $20–26 billion. Additionally, indirect costs may pose a serious burden (e.g. loss of productivity, costs of disability pensions, sick leaves, and premature mortality), reaching in some countries almost the level of medical and non-medical direct costs (Hoogendoorn et al. 2014; Jahnz-Różyk 2009).

The present study demonstrates that annual expenses incurred by the Social Insurance Institution toward benefits associated with incapacity for work due to COPD amounted to €56.4 million in 2012. This sum represented 0.78 % of the total expenditure incurred by the Social Insurance Institution in regard to all diseases. Of the amount, 89.3 % were pensions due to incapacity for work, 7.9 % were benefits paid in connection with sickness absenteeism, 1.9 % were rehabilitation benefits, 0.48 % were expenses incurred in connection with medical rehabilitation, while 0.4 % – social assistance benefits. Pensions due to permanent incapacity for work due to COPD were received by 19,100 beneficiaries in a total amount of €50.4 million, which makes it the predominant chunk of the expenditure above outlined.

The fact that every second person with COPD is of working age remains a broad social and health care problem (Chazan 2013). According to the BMC Public Health report (Fletcher et al. 2011), 40 % of working people with COPD takes up a disability pension each year

due to disease progression; the average age of these persons is less than 54 years. The results of studies carried out in Brazil, China, Germany, Turkey, US, and the UK (Fletcher et al. 2011) show that at the average annual treatment cost of $2364, the cost of lost workhours is $880, not taking into account the costs of taking up early retirement, lost professional careers, or those incurred by family members to exercise extra care for the sick. The costs of lost revenue alone are estimated at more than $7000, where differences in individual countries are substantial– from several hundred to several thousand US dollars.

A low percentage (0.2–1.4 %) of expenses incurred by the Social Insurance Institution due to incapacity for work attributable to COPD, against expenditures resulting from other diseases, particularly cardiovascular diseases or cancer, as well as certificates and statements issued on account of COPD against the total number of certificates issued due to other diseases, actually demonstrates a low rate of diagnosis of COPD and a substantial underestimation of the problem.

In the present study, expenses incurred by the National Health Fund in connection with the incidence of COPD under the framework of prevention of tobacco-related diseases were at a comparatively low 0.8 %. Medical advice provided to the 0–65 age group of patients accounted for about 80 %, while that in 65+ group was less than 20 %. Since, generally, smoking remains the major risk factor for COPD, quitting smoking is the basis of prevention (Śliwiński et al. 2014; Nizankowska-Mogilnicka et al. 2007) and it also is a life-prolonging procedure in patients already having COPD. Expenses incurred for medical rehabilitation also were at a small percentage level. An Australian study has demonstrated that a large proportion of patients with COPD do not decide to make use of a rehabilitation program or do not finish it up (Keating et al. 2011). Reluctance to participate in rehabilitation stems from a conviction of its low efficiency in improving health, with the simultaneous discomfort related to its execution. That is a matter of proper health education to make the patients more often participate in rehabilitation, which definitely is of distinct health and quality of life benefits in COPD.

We identified differences in the value and number of settled benefits or the number of patients between individual voivodeships. Such differences might have to do with the number of active pulmonologists and the quality of medical providers which have contracts with the public payer. Regional health policy for prevention, diagnosis, and treatment of COPD differs, which influences the number of cases and the public spending for health. Low indicators in the category of social welfare depended mostly on high unemployment rates in particular regions (Central Statistical Office 2013). Regional variations have been observed in European countries in other reports (López-Campos et al. 2013). Differences were due mainly to disparate standards of health care offered to patients by local hospitals, outpatient clinics, and of access to pulmonologists. Admission rates for COPD patients differ as much as 10-fold between European countries (European Lung White Book 2013). Clear differences between regions also are shown by the Nordic COPD Index 2010, which compares COPD management in 20 regions of the four Nordic countries (Lindblad and Björnberg 2010).

The WHO recommends the implementation of a comprehensive, integrated system of care for patients with COPD as the most effective way of dealing with the disease. It is advocated to create an electronic database of patients, managed by a regional coordinator and accessible to the entire team caring for the patient (Jassem et al. 2010). In view of the projected increase in the incidence of COPD, economic and social costs will rise. The issue of COPD is still insufficiently studied and the optimization of procedures remains to be found. Primary health care is in possession of a diagnostic tool for lung diseases, i.e., pulmonary function tests. Performing spirometry with a bronchodilator trial should be the gold standard in the diagnosis and implementation of appropriate treatment

(Russell and Norcliffe 2008). Foremost, spirometry would identify patients with COPD whose disease has by far been missed. Extensive social actions, such as Spirometry Days (Dąbrowiecki et al. 2013) where the news on COPD and it diagnosis is widely spread by public mass media, raise the knowledge on the disease, and the role of cigarette smoking and environmental pollution in its pathomechanisms, and appear to be an effective preventive measure.

Multiannual strategies are one of the main tools for shaping and implementing health care for chronic diseases. Therefore, they are used by a number of European Union countries, the experience of which shows that such plans are conducive to the achievement of rapid progress in the areas of health protection regarded as priority (Knai et al. 2012; Decramer et al. 2011). The strategy should define priority objectives and projected health care benefits, in particular: it should ensure equal access to benefits, determine the competence of hospital and primary care services, and set diagnostic standards and optimal models of pharmacotherapy and prevention. Changes in the way of financing general practitioners in connection with the treatment of patients with COPD are also required. The financing should be conditioned on the implementation of specific procedures and effects of therapy, e.g., a decrease in the number of hospitalization. Correct diagnosis of COPD, and a timely application of treatment would benefit patients, delay disease progression, and reduce health care and social costs. Finally, reduction costs associated with sickness absenteeism and benefits for temporary incapacity for work requires developing a disability pension program for COPD patients. Such a program, in association with rehabilitation, psychological support and vocational guidance, would further improve the overall health of patients and their functioning in social and professional life.

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

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Costs of Treatment of Chronic Obstructive Pulmonary Disease

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Abstract

The aim of this study was to analyze direct costs of COPD therapy in relation with clinical course and stage of the disease. Sixty patients with moderate to severe COPD were included into the study. The average cost was taken from institutional data file and was also assessed from a social perspective. Results were presented as average costs per patient per year. Forty two percent of patients was classified as GOLD D category, while categories A, B, and C accounted for 8 %, 27 %, and 23 %, respectively. Approximately 65 % of patients had 2–3 degrees of dyspnea according to the Modified Medical Research Council Dyspnea Scale. About 60 % of patients underwent two or three exacerbations per year and those patients had one or two co-morbidities diagnosed. Treatment costs almost doubled with disease progression, mainly due to exacerbations. In patients in Group C and Group D with exacerbations the direct costs were several times higher than in group A or B and the difference increased with progression of the disease. In Groups A and B, the costs of treatment of stable disease or with exacerbation were comparable. We conclude that costs of treatment of COPD patients were highest in advanced disease and were strongly related to COPD exacerbations.

Keywords

COPD • Direct costs • Exacerbations • Medical costs • Pharmacoeconomics

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1 Introduction

COPD is a preventable and treatable disease that is characterized by partially irreversible airflow limitation of airways. The disease is usually progressive and associated with abnormal responses of lungs to noxious particles or gases (GOLD 2011). COPD is currently the fourth leading cause of death among adult patients globally, and it is projected that it will be the third most common cause of death by 2020. Between 1970 and 2002, in the US the percentage of deaths due to all-cause cardio-vascular mortality decreased from 63 to 52 %, while due to COPD it increased by 100 % (Jemal et al. 2005).

High incidence and mortality of COPD substantially raise the financial burden for the state budget (Pauwels et al. 2004). According to the annual report of the World Health Organization (WHO 2011), COPD affects approximately 600 million people. Each year, COPD causes the deaths of about three million people. COPD touches 9–10 % of Europeans ≥40 years of age (Mannino 2003). In the EU, the costs of respiratory diseases are estimated at 6 % of the budget allocated to health care. Of this amount, 56 % is allocated for the treatment of COPD patients. This generates a cost of about € 38.7 billion annually, and causes the loss of 66,155 working days per 100,000 population, which represents 62.4 % of the total work-related absenteeism. A total COPD-related cost in the US is estimated at $ 36.1 billion, of which 20.9 billion are direct costs (GOLD 2011; Mannino 2003).

It is estimated that in Poland about two million people suffer from the disease, with 80 % experiencing mild-to-moderate and 20 % severe and very severe stage of the disease. COPD is more common in men (11 %) than women (5 %) (Halbert et al. 2006). The number of hospitalized COPD patients in Poland is estimated at 36 %. Studies show that one per ten Poles over 30 year of age have COPD symptoms. Each year, around 4 % of all hospitalizations are due to COPD. It is a common cause of sick leave, invalidity, and premature death. Life annuities are granted to patients between 50 and 60 years of age, which means lower productivity and increasing direct and indirect costs of treatment. Annually, about 15,000 Poles die from COPD.

In 2011 year, a group of experts from GOLD proposed a new classification of COPD, taking into account the severity of the airflow limitation, the number of symptoms, and the risk of COPD exacerbations (GOLD 2011). The severity of COPD symptoms was assessed with the Modified Medical Research Council Dyspnea Scale (mMRC) (Doherty et al. 2006) and with the COPD Assessment Test (CAT) (Curkendall et al 2006). These assessments allows creating four categories of COPD evaluation. COPD exacerbation is a sudden worsening of the clinical condition of the patient, which manifests as shortness of breath and coughing, and a change in the nature and volume of sputum. It takes at least 24 h until the patients is forced to seek medical attention and modifies treatment (Caramori et al. 2009; Pauwels et al. 2004). COPD exacerbations increase hospitalization rates, and affect the quality and duration of the patient's life. According to the recent GOLD recommendations, the number of exacerbations undergone in the past year, appears to be a good predictor of the risk of exacerbation, which should be included in the algorithms for diagnosis and therapeutic intervention (Hurst et al. 2010). The objective of treatment of COPD exacerbations is to minimize the health deterioration and to prevent subsequent exacerbations (Sin et al. 2005). Over 80 % of exacerbations can be treated on the outpatient basis using bronchodilators, corticosteroids, and antibiotics (Hurst et al. 2010; Celli et al. 2008; Tashkin et al. 2008). The therapeutic approach is based on the GOLD A–D category classification, aiming to improve airway function, reduce the inflammatory process, and to facilitate coughing up sputum. A study by Hilleman et al. (2000) shows that exacerbations that are treated in the hospital represent 70 % of direct medical costs. In Poland, the average cost of treating COPD exacerbation in the hospital is close to € 1200.

The aim of the present study was to analyze direct costs of COPD therapy in relation with clinical course and stage of the disease.

2 Methods

2.1 Subjects and Treatments

The study was approved by the Institutional Ethics Committee. This a retrospective study that was conducted among 60 patients with moderate and severe COPD, treated in the Respiratory Medicine Center in Bialystok, Poland, during the period of January 2012–December 2012. The study included patients diagnosed and treated according to the GOLD criteria for COPD, which had started at least 30 days before the onset of a current exacerbation (GOLD 2011). Likewise, disease severity was assessed according to the GOLD criteria; the patients were stratified into four categories of increasing severity: A, B, C, and D. Exacerbations of COPD were evaluated according to the classification of Anthonisen (Calverley et al. 2009). The five-point mMRC dyspnea scale was used to judge the perception of dyspnea associated with daily activities. The estimations of the costs of treatment of stable forms of the disease and of exacerbations were conducted from a societal perspective. The direct costs included drugs, diagnostic tests, and ambulatory care. The costs were derived from the existing data of the Polish National Health Fund and the Ministry of Health as of 2012. The number of exacerbations in each category of COPD patients is presented in Table 1.

2.2 Statistical Elaboration

Descriptive statistics were used to describe the basic features of the study population and of the data on COPD treatment and costs involved. The categorical data were expressed in proportion, while continuous data were expressed as means ± SE. The significance of differences was tested using the nonparametric Mann-Whitney U test. The use of health care resources and the individual cost components of the disease are presented as the average cost *per* patient *per* year; 95 % confidence intervals (95 % CI) were calculated using the method of bootstrapping. A p-value <0.05 was considered statistically significant. In the case of data aggregation confidence intervals were not determined.

3 Results

The description of the COPD patients investigated is presented in Table 1. Stratification of patients into COPD categories shown in this table concerns the time of the enrolment into the study. During a one year period of treatment the patients conditions changed, which necessitated relocation of some patients into different categories. The final results of changed patients' allocation, along with the number of exacerbations in a given category, are presented in Table 2.

The feeling of breathlessness was increasing in COPD patients with increasing level of disease severity. The mean data of dyspnea score, as assessed in the mMRC scale, are shown in Fig. 1. We found significant differences between

Table 1 Characteristics of COPD patients

	COPD A	COPD B	COPD C	COPD D
No. of patients (%)	3 (5.0)	4 (6.7)	37 (61.6)	16 (26.7)
Age	63.3 ± 5.4	72.7 ± 7.9	67.8 ± 8.3	65.7 ± 5.4
Pack/years	39.3 ± 18.5	43.1 ± 17.7	45.5 ± 23.7	43.5 ± 22.7
FEV1 %pred.	62.3 ± 11.4	42.1 ± 10.1	30.2 ± 10.2	26.1 ± 6.1
FEV1	2.1 ± 0.4	1.3 ± 0.3	0.9 ± 0.2	0.7 ± 0.1

COPD categories A, B, C, D; FEV_1 – forced expiratory volume in 1 s; pack/years – the number of packs of cigarettes smoked per day multiplied by the number of years the person has smoked

Table 2 Number of exacerbations in each category of COPD patients after a year's treatment

	COPD A	COPD B	COPD C	COPD D
No. of patients (%)	5 (8.3)	16 (26.7)	14 (23.3)	25 (41.7)
No. of exacerbations/per year	1	1	2	8

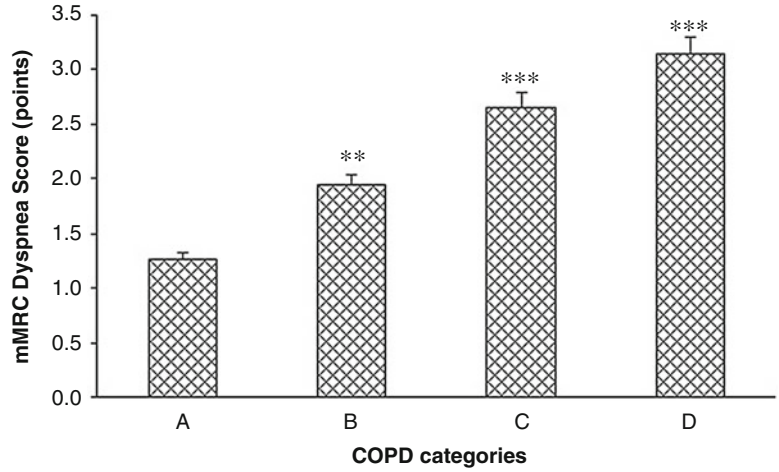

Fig. 1 mMRC dyspnea score in different COPD categories. Data are means ± SE. See description of statistically significant differences in text

category A of COPD and the remaining categories regarding the dyspnea score: A *vs*. B (**p = 0.005), A *vs*. C (***p = 0.0001), and A *vs*. D (***p = 0.0001). The differences between other COPD categories were inappreciable. The higher the dyspnea score the greater is the risk of exacerbation according to GOLD initiative (GOLD 2011).

The forced expiratory flow in 1 s (FEV_1) decreased with increasing mMRC dyspnea score. There were significant differences between FEV_1 at each score mark of mMRC dyspnea scale; FEV1 at 2–3 points *vs*. 0–1 point (**p < 0.005), FEV1 at ≥4 points *vs*. 0–1 point (***p < 0.0001), and FEV1 at ≥4 points *vs*. 2–3 points ($^{++}$p < 0.005) (Fig. 2).

Costs of treatment of COPD exacerbations presented against those of stable COPD maintenance treatment are presented in Table 3.

Direct health care costs included those related to medical visits to a specialist clinic for patients in each COPD category with both stable disease and with disease exacerbations. The mean costs of medical services for patients with stable and exacerbated COPD, calculated per patient per year are presented in Fig. 3. The costs were significantly higher for the disease with exacerbations (p < 0.05) and were steadily increasing along with the disease severity. These costs were concerned with type I, II, and III medical services. Service type I is an examination without any specialized diagnostic tests, type II is a visit to an outpatient facility combined with a spirometry examination, and type III is a visit with spirometry and X-ray examinations. The costs of procedures were based on the evaluation set in a contract signed between a given specialist clinic and the Polish National Health Fund. In COPD category A, stable patients had spirometry done one time over the year and three times benefited from the provision of specialized medical visit. In case of exacerbations, the annual cost of treatment was increased due to one type III visit (including spirometry and X-ray examinations). In COPD category B, the number of spirometry examinations performed during 1 year was similar, but patients had 5 more visits to a specialist clinic than those in category A. In this case, the annual cost of treatment was increased by one

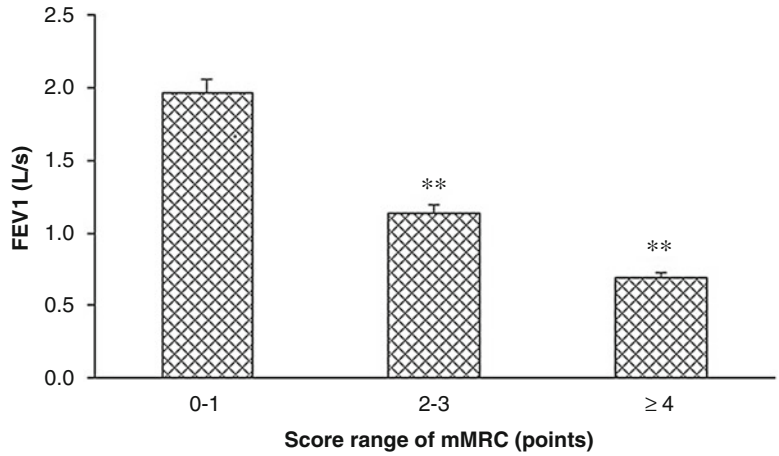

Fig. 2 Forced expiratory volume in 1 s (FEV_1) *vs.* score ranges of the modified Medical Research Council Dyspnea Scale (mMRC) in COPD patients. Data are means ± SE; see description of statistically significant differences in text

provision of specialized type III visit. In the stable phase of the disease, patients from categories C and D had two spirometry examinations and four medical visits per year. In case of exacerbations in category C, patients had spirometry and X-ray examinations performed twice. In category D, each exacerbations was linked to a medical visit type III, i.e., with spirometry and X-ray examinations, with the costs increasing accordingly.

4 Discussion

Recent GOLD recommendations define new algorithms for the treatment of COPD (GOLD 2011). In contrast to previous recommendations, recent guidelines go far beyond medical treatment. The guidelines especially focus on intensive and preventive care. Such approach may result in a reduction of symptoms of chronic and exacerbated disease, improved prognosis, and improved quality and length of life. Such changes would obviously relate to the economic aspects as well. COPD is accompanied by chronic inflammation, which in contrast to asthma responds poorly to inhaled glucocorticoid therapy. Moreover, during exacerbations of the disease caused by unpredictable and poorly controlled factors, a dramatic change for the worse of inflammatory status may be observed (World Health Statistics 2011). Deterioration of clinical condition requires changes in treatment modalities and further increases pharmacotherapy costs. Improvement in quality of life of patients, their extended presence in the labor market, and a reduced number of hospitalizations would mitigate the economic burden of the disease.

There are different regulations concerning refinancing of medical services in different countries. Therefore, detailed economic analyses should be carried out at national levels, which could be later integrated into the multicenter assessments in the EU to get insight into the scale of the issue at the cross-national level. In recent years, published data regarding the cost analysis of COPD treatment have been relatively few. The reason for that may be an established dominance of conservative pharmacotherapy and associated lack of measurable long-term outcomes. The disease, however, has improved prognosis of late, which is clearly related to changes in the algorithms of treatment. In the EU in 2011, a total expenditure related to the treatment of COPD amounted to €141.4 billion, which included €23.3 billion in direct costs and €25.1 billion in indirect costs concerned with lost productivity. The costs of €1.69 million per year of disability-adjusted life-years (DALYs) in COPD totaled €93 billion. Direct costs of drugs accounted for €7.1 billion, outpatient care for €8.9 billion, and hospitalizations for €7.3 billion (World Health Statistics 2011).

Table 3 A breakdown of costs of chronic obstructive pulmonary disease (COPD) pharmacotherapy in a stable disease phase

Stable COPD			Exacerbations of COPD		
Medicines	Cost of medicines/ patient/ year	Grand total cost of medicines/ group of patients/year	Type of treatment	Cost of medicines/ patient/ year	Grand total cost of medicines/ group of patients/year
	EUR	EUR		EUR	EUR
Category A					
Salbutamol 0.10 mg/dose	39.86	119.57	Amoxicilin/ clavulanate 0.875/0.125 g	47.66	238.30
Ipatropium bromide 0.02 mg/dose	73.09	219.26	or		
Ipatropium bromide 0.25 mg/ml	37.23	111.69	Clarithromycin 500 mg	45.03	225.15
Category B					
Formoterol 0.012 mg/dose	184.46	737.83	Amoxicilin/ clavulanate 0.875/0.125 g	182.55	2920.80
Salmeterol 0.05 mg/dose	176.40	705.60	or		
			Clarithromycin 500 mg	184.20	2947.27
Category C					
Tiotropium bromide 0.018 mg/dose	421.57	15,598.14		437.18	6120.47
Budesonide 0.08 mg/dose, Formoterol 4.5 µg/dose	217,86	8060.71		233.46	3268.47
Budesonide 0.16 mg/dose, Formoterol 4.5 µg/dose	251.23	9295.46		266.83	37,353.67
Budesonide 0.32 mg/dose, Formoterole 9.0 µg/dose	487.80	18,048.60		503.44	7047.67
Fluticasone propionate 0.05 mg/dose, Salmeterol 0.025 mg/dose	268,54	9936.09	Amoxicilin/ clavulanate 0.875/0.125 g	284.15	3978.07
			or		
			Clarithromycin 500 mg		
Fluticasone propionate 0.125 mg/dose, Salmeterol 0.025 mg/dose	344.51	12,747.03		360.12	5041.67
Fluticasone propionate 0.25 mg/dose, Salmeterol 0.025 mg/dose	510,71	18,896.43		526.32	7368.48
Fluticasone propionate 0.10 mg/dose	291.57	10,788.14		307.18	4300.52
Salmeterol 0.05 mg/dose					
Fluticasone propionate 0.25 mg/dose	366.23	13,550.46		381.83	5345.67
Salmeterol 0.05 mg/dose					
Fluticasone propionate 0.5 mg/dose	481.51	17,816.03		497.12	6959.67
Salmeterol 0.05 mg/dose					

(continued)

Table 3 (continued)

Stable COPD			Exacerbations of COPD		
Category D					
Tiotropium bromide 0.018 mg/dose + Formoterol 0.012 mg/dose	610.87	9773.95		618.18	13,599.99
Tiotropium bromide 0.018 mg/dose + Budesonide 0.16 mg/dose, Formoterol 4.5 μg/dose	671.675	10,764.80		648.84	15,454.0
Tiotropium bromide 0.018 mg/dose + Fluticasone propionate 0.125 mg/dose, Salmeterolol 0.025 mg/dose	766.09	12,257.37	Amoxicilin/Clavulanate 0.875/0.125 g or Clarithromycin 500 mg	696.21	17,405.25
Tiotropium bromide 0.018 mg/dose + Fluticasone propionate 0.25 mg/dose, Salmeterol 0.05 mg/dose	787.80	12,604.80		789.49	19,737.25
Tiotropium bromide 0.018 mg/dose + Budesonide 0.16 mg/dose, Formoterol 4.5 μg/dose + Fluticasone propionate 0.25 mg/dose, Salmeterol 0.05 mg/dose	1090.46	17,447.31		1113.86	27,846.5

A, B, C, and D – categories of severity of COPD according to GOLD criteria (GOLD 2011). All costs listed above include a possible partial repayment by the National Health Fund; '+' denotes a mix of two drugs in one container

The present study included patients with varying degrees of severity of COPD, but the largest group of patients was allocated to group C with the dyspnea mMRC score of 2–3 points. We found that the direct cost of treatment increased in harmony with an increased number of COPD exacerbations occurring during a year of observation. Moreover, costs of COPD treatment of patients in early stages of both stable and exacerbated disease are comparable. Although the number of exacerbations and the total costs increased with increasing severity of the disease, the antibiotic use for exacerbation treatment did not appreciably affect the overall annual cost of pharmacotherapy. These data are consistent with the findings of others (Mapel and Roberts 2012; Vázquez-Polo et al. 2011).

Published data regarding costs of COPD treatment come mostly from selected groups of patients. The selection of patients is preferably based on clinical advancement of the disease and the cost analysis is based on direct costs of medical procedures. The aim of such approach has been to predict the frequency of exacerbations (Starkie et al. 2008). One of the most important parameters regarding pharmacoeconomics is the hospitalization rate. Data derived from studies performed in the developed countries show that the hospitalization rate varies from 27 to 43 patients per 100 patients with COPD. That indicates a substantial health care and social costs of exacerbations (Kopec and Willison 2003). The costs linked to taking care of COPD patients are lower in Poland compared with those in the US where the total societal cost is $5646 per patient per year (Wouters 2003), which may be related to differences in health care systems. That does not change the fact that the disease is a major health and social issue and the costs are extremely high in both countries (Jahnz-Różyk et al. 2011b).

In Poland in 2008, the annual cost of treating a COPD patient on an outpatient basis amounted to nearly €1000, where symptomatic therapy accounted for €600, exacerbations treatment €100, and approximately €300 was laid out for post-exacerbation hospitalizations (Jahnz-Różyk et al. 2011b). Therapy for patients with

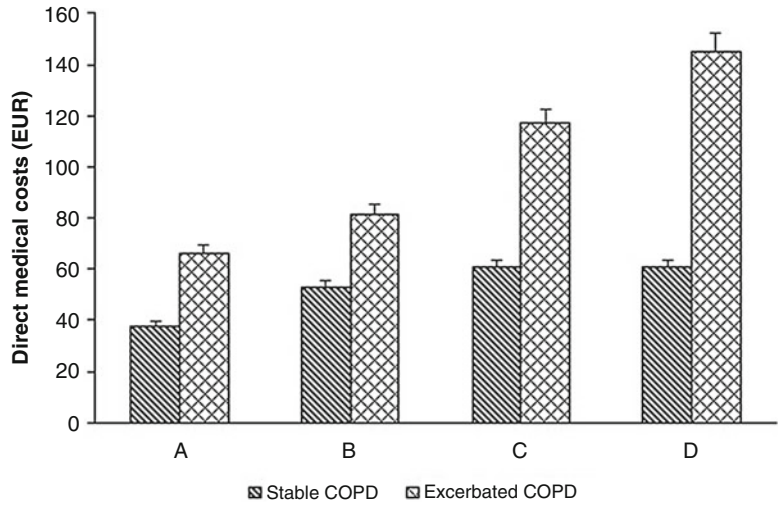

Fig. 3 Direct medical costs of caring for COPD patients with stable and exacerbated disease

exacerbations was substantially more expensive than the corresponding costs for patients in a stable condition. The cost gap significantly increased in case of severe COPD, which might be due to the use of different medical procedures. The same group of authors in another study compares the cost of treatment of COPD exacerbations in patients with varying degrees of severity of the disease. The costs of treatment of exacerbations requiring hospitalization amounted to €1000 per patient, which means that inpatient treatment of COPD exacerbation requires a 6-fold higher budget than that needed for outpatient treatment (Jahnz-Różyk et al. 2011a). In the present study, direct costs increased due primarily to the use of specialized services and specific diagnostic procedures, which mostly concerned the treatment of patients with severe disease progression (groups C and D). The costs reported in the present study differ from those reported from other countries (D'Souza et al. 2006). An explanation for the relatively high cost of exacerbation treatment may be a delayed diagnosis of COPD exacerbations made in Poland. On the other hand, a higher average age of Polish COPD patients, close to retirement age, reduces indirect costs of caring for COPD patients.

However, since indirect costs positively correlate with disease severity, treatment cost of severe form of COPD is about 4-fold higher compared with that of milder stages. This observation is also valid when the new GOLD strategy of A–D categories is applied. The analysis of indirect costs in a randomized study of a large group of COPD patients treated with fluticasone has demonstrated a cost-effective decrease in the number of exacerbations, meaning that the monetary savings were sufficient to compensate for the increased cost of treatment (Ayres et al. 2003). That also underscores the importance of prophylactic counteracting the appearance of exacerbations in the course of COPD, which may decrease the overall costs.

In summary, the present study indicates that direct costs of treating COPD increase with the number of exacerbations per year. This highlights the need for a strict implementation of preventive measures in this regard. In case of milder disease forms, treatment costs are relatively small, regardless of exacerbations. A small number of cases in our study requires further assessment of multicenter study. It is worthy of note that a high number of hospitalizations due to COPD exacerbations in Poland dramatically increase direct medical costs. The situation may be improved by adherence to the new GOLD strategy concerning the treatment of COPD exacerbations.

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Coexpression of Galanin and Nestin in the Chemoreceptor Cells of the Human Carotid Body

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Abstract

The carotid body is a highly specialized chemoreceptive organ of neural crest origin whose role is to detect changes in arterial oxygen content. The sensory units are the chemoreceptor cells, which are neuronal-like cells, surrounded by sustentacular or glial-like cells. It is suggested that the carotid body contains self-renewing multipotent stem cells, which are putatively represented by glial-like sustentacular cells. The mechanisms of renewal of neuronal-like cells are unclear. Recently, we have demonstrated the expression of galanin, a peptide promoting neurogenesis, in chemoreceptor cells in the human CB. Thus, in the present study we seek to determine whether galanin expression in chemoreceptor cells could be matched with that of nestin, a peptide that is a marker of multipotent neural stem cells, or rather with the glial fibrillary acidic protein (*GFAP*), a marker for glial cells. The latter would underscore the pluasibly essential role of sustentacular cells in the self-renewal capability of chemorecetors. We found that galanin expression is matched with nestin in chemoreceptor cells of the human carotid body, but not with that of GFAP. Thus, galanin expression in chemoreceptor cells could provide a signal for neurogenesis and chemoreceptor cell differentiation in the carotid body.

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Keywords
Carotid body • Chemoreceptor cells • Galanin • Hypoxia • Nestin • Neurogenesis

1 Introduction

The carotid body (CB) is a paired arterial chemosensitive organ devoted to acute oxygen-sensing and oxygen homeostasis. The organ generates the stimulatory hypoxic ventilatory response to hypoxia. The CB originates from the neural crest, has a lobular organization, with the lobes separated by thin connective septa. The CB chemosensing core constitutes an intricate network of blood vessels that originate from a branch of the external carotid artery, and which spread into the parenchyma of the CB. The chemosensitive units consist of glomus or type I or chemoreceptor cells, which are neuronal-like cells, surrounded by Type II or sustentacular cells (López-Barneo et al. 2008; Prabhakar 2000; 2006; Verna 1979). The sustentacular cells are glial-like cells that have a supportive role and express the glial fibrillary acidic protein (GFAP) (Pallot 1987). Chemoreceptor cells release a number of neurotransmitters and neuromodulators in response to stimulating conditions (Porzionato et al. 2008; Shirahata et al. 2007; Nurse 2005; Iturriaga and Alcayaga 2004). This, in turn, elicits afferent discharge in the sinus nerve, which is conveyed through the glossopharyngeal nerve to the petrosal ganglion (Iturriaga et al. 2007).

CB tissue undergoes modifications with age; the connective tissue compartment increases, which results in a reduction of sensory elements. Analogous changes have been reported in young drug-addicted subjects (Zara et al. 2013; Porzionato et al. 2005; Di Giulio et al. 2003). But the opposite process, enlargement of sensory elements, is possible. The organ enlarges to several-fold its normal size in chronic hypoxia, which also is seen in patients with cardiopulmonary disease (Wang and Bisgard 2002; McGregor et al. 1984; Heath et al. 1982). This occurs through the production of new neuronal-like cells by activation of a resident population of neural crest-derived progenitor cells. Further, on returning to normoxic condition, the original size of the CB is restored, with about half of chemoreceptor cell mass replaced by the newly formed cells, which indicates a striking regenerative power. The newly formed cells have the same neurochemical composure and electrophysiological properties as the indigenous glomus cells. Consequently, the CB has been considered a neurogenic center with a distinct physiological function in adult life. It is suggested that the organ contains self-renewing multipotent stem cells, which are putatively represented by glial cells (Pardal et al. 2007). However, the exact mechanisms of renewal of neuronal-like cells remain unclear. Recently, we have demonstrated the expression of galanin in neuronal-like, chemoreceptor cells in the human CB (Mazzatenta et al. 2014). Galanin is a neurotrophic/neuroprotective peptide involved in neural plasticity. This peptide upregulates genes involved in neuronal signaling pathways, and neuronal differentiation. It also promotes neurogenesis (Cordeo-Llana et al. 2014; Ma et al. 2008). Therefore, in the present study we set out to examine whether galanin expression could be matched with that of nestin, a peptide that is conducive to the proper self-renewal of neural stem cells (Park et al. 2010) or rather with the GFAP peptide, which would underscore the suggested essential role of glial-like cells, above outlined, in the self-renewal of cells in the CB.

2 Methods

The study was approved by a local Ethics Committee and was performed according to the

Italian law on the use of human autopsy tissue. Human carotid bodies (n = 12) were collected from 12 to 72 h *post-mortem* (the age range of deceased subjects was 27–76 years). The exclusion criteria were signs of past cardiac hypertrophy, myocardial infarction found during autopsy, history of other chronic pulmonary or cardiovascular disease, and degenerative tissue alterations detected in histologically stained tissue specimens. Further, the possible influence of the death-to-autopsy interval on the state of tissue specimens was examined by way of a statistical method (for details see Porzionato et al. 2005).

The specimens were fixed in 10 % formalin, embedded in paraffin, sectioned (3 μ thick), subjected to the Mallory trichrome histological staining (Bio Optica; Milan, Italy). The immunohistochemistry consisted of the following: H-11 mouse monoclonal anti-galanin antibody (sc166431; Biotechnology; Santa Cruz, CA), H1α 67 anti-hypoxia inducible factor (HIF-1 α) antibody (sc-53546; Biotechnology; Santa Cruz, CA), and the developing kits (HRP Polymer/DAB Plus Chromogen and Lab Vision UltraVision LP Detection System; Thermo Fisher Scientific, Carlsbad, CA). Serial sections were examined under light microscopy (Leica DM 4000 microscope), equipped with Leica DFC 320 digital acquisition system. The QWin Plus 3.5 software (Leica, Cambridge, UK) was used to digitalize images and to compute areas positive for antibodies. Statistical analysis employed one-way ANOVA, with α set at 0.001, unless otherwise specified. Commercial SPSS and Origin packets were used for statistical analysis.

3 Results

Figure 1, panels A and B, demonstrates the anatomical organization of the human CB. There are clusters of chemoreceptor cells, surrounded peripherally by connective tissue. The clusters are cut through by a network of capillaries. The anatomical structure highlighted in the histological staining revealed fairly well preserved CB parenchyma despite dealing with autopsized tissue. Panels C and D of the figure demonstrate immunohistochemical staining for HIF-1 alpha in chemoreceptor cells, which was used for the verification of visualization of these cells.

The immunohistochemical investigation of the human carotid body parenchyma yielded the following findings. The expression of galanin and nestin was found in chemoreceptor cells, whereas that of GFAP in sustentacular cells surrounding the clusters of the former cells (Fig. 2A, B, and C). Densitometry, a quantitative measurement of optical density of immunostaining expressed as the percentage of positively stained area, yielded confirmatory evidence for the coexpression of galanin and nestin in chemoreceptor cells (one-way ANOVA pointed to insignificant differences between the two neuropeptide expression; $F_{(1.10)} = 1.03$; $p = 0.33$), while there was significantly different expression between galanin and GFAP ($F_{(1.10)} = 5.3$; $p < 0.05$).

4 Discussion

As shown by Pardal et al. (2007), nestin and GFAP are markers of differentiating new neuronal-like and glial-like cells, respectively. In the present study we investigated the hypothesis that galanin could be involved in the mechanism of neuronal-like differentiation in the CB. We addressed the issue in an immunohistochemical examination performed in serial sections of CB parenchyma, in which we sought to determine whether galanin would colocalize with nestin or rather with GFAP. We found that the expression of galanin colocalizes with that of nestin in chemoreceptor cells, but not with GFAP. The immunohistochemical findings were strengthened by quantitative densitometry, which pointed to the lack of difference between the expressions of galanin and nestin, and to an appreciable difference between galanin and GFAP.

Galanin expression has been shown throughout both central and peripheral nervous systems, including the CB of rat, monkey, guinea pig,

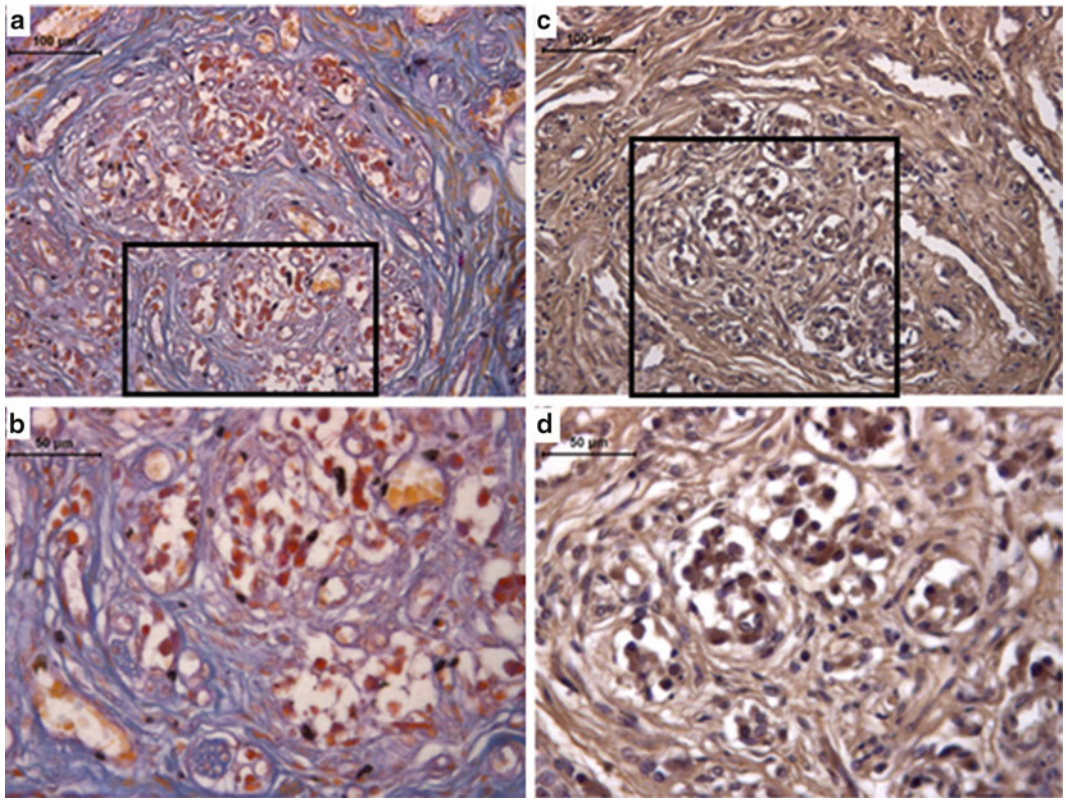

Fig. 1 **Panels A** and **B** – anatomical structure of a human autopsized carotid body, stained with the Mallory trichrome technique; **panels C** and **D** – immunohistochemistry of HIF-1 alpha expression in chemoreceptor cells, taken as their marker. **Panels B** and **D** show doubly magnified parts of the clusters of chemoreceptor cells surrounded by connective septa as outlined by rectangles in the corresponding *upper panels*

chicken, and man (Mazzatenta et al. 2014; Finley et al. 1995; Ichikawa and Helke 1993; Kameda 1989; Heym and Kummer 1989). This neuropeptide in animals consists of 29 amino acids, while it has 30 amino acids in man. It is a highly inducible neuropeptide that is upregulated in the nervous system after pathological disturbance, where its N-terminal region is crucial for biological activity (Branchek et al. 2000). Biological effects of galanin are mediated through three different G-protein coupled receptors: GalR1, GalR2, and GalR3 (Branchek et al. 2000; Florén et al. 2000). The GalR3 has not been detected in carotid chemoreceptor cells in the rat (Porzionato et al. 2010), although it is present in the rat brain (Smith et al. 1998). The GalR1 sustains a transduction cascade through inhibition of adenylyl cyclase, while the GalR2 acts through the activation of phospholipase C and protein kinase C. Therefore, galanin can regulate differentiating neural cells and it participates in the development and regulation of plasticity of the nervous system (Wittau et al. 2000; Wang et al. 1998).

The CB undergoes size adaptation in chronic hypoxia, which leads to an increase in the organ's volume, due to the formation of new neuronal-like, chemoreceptor cells. Conversely, when returned to normoxia, there is a corresponding decrease in CB size. The sensory cells can be renewed by the activation of a resident population of neural-crest-derived progenitors (Wang and Bisgard 2002; McGregor et al. 1984; Heath et al. 1982). These adaptive phenomena point to the probable presence of self-renewing, multipotent stem cells in the CB

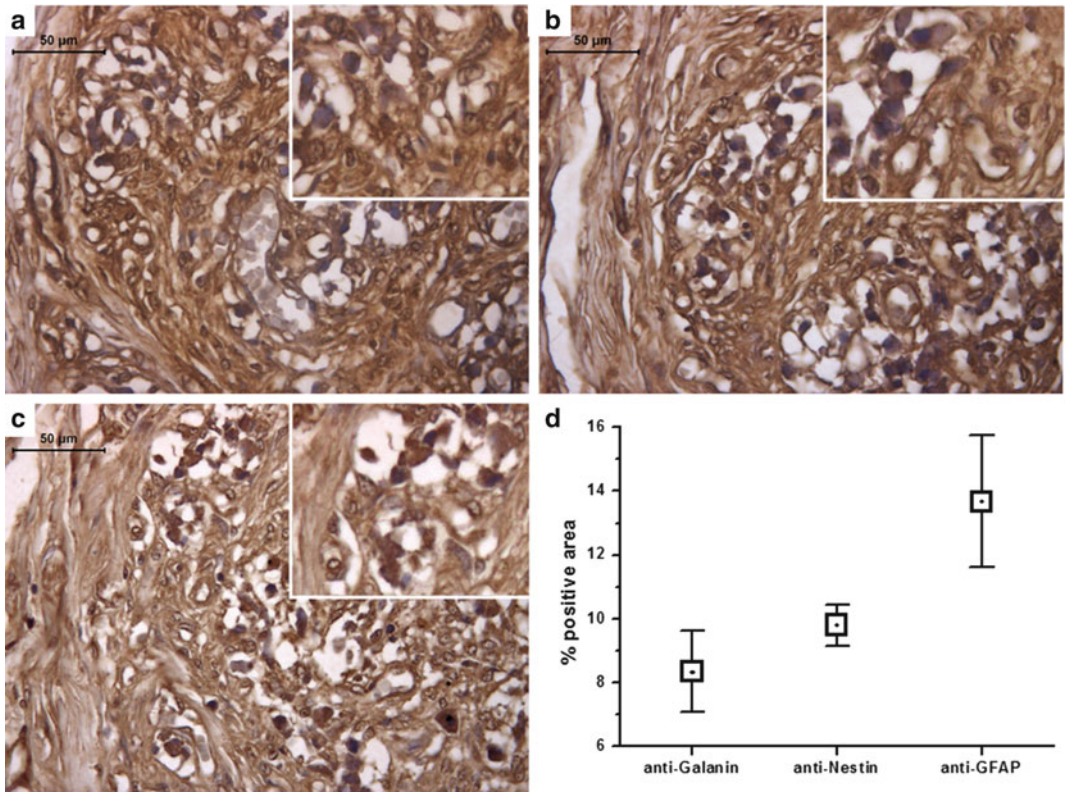

Fig. 2 **Panel A** – expression of galanin in carotid chemoreceptor cells; **panel B** – expression of nestin in carotid chemoreceptor cells, and **panel C** – expression of glial fibrillary acidic protein (*GFAP*) in sustentacular cells of the human carotid body. Insets in all three panel show zoomed in details. **Panel D** – comparison of densitometry assessment of immunostaining of the three neuropeptides in the human carotid body

(Pardal et al. 2007). The ability for self-renewing seems crucial for the physiological role of the CB, since in adult life it enables to maintain a population of cells that can differentiate into neuronal-like cells on demand (Mazzatenta et al. 2014).

In conclusion, we believe we have shown that both galanin and nestin are coexpressed in a population of neuronal-like, chemoreceptor cells in the human CB. The finding reinforces our hypothesis that galanin could be involved in the mechanism of chemoreceptor cell differentiation, rather than glial fibrillary acidic protein present in sustentacular glial-like cells.

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

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Prosthetic Rehabilitation of Patients After Surgical Treatment of Maxillary Tumors with Respect to Upper Airway Protection

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Abstract

As a consequence of surgical treatment of maxillary tumors, a connection between oral and nasal cavities is formed, which leads to serious functional disorders, manifested by inability to normally ingest food, proper speech articulation, and to respiratory route disorders and upper airway inflammation. These morphological and functional disorders are intensified by adjunctive radio- or chemotherapy. The aim of this paper is to present different possible methods of rehabilitation, including application of interim obturators and individually planned prosthetic restorations to improve respiratory efficiency in patients after extensive maxillary resections. In the course of prosthetic treatment, cooperation with the laryngologist to consider every aspect of chronic paranasal sinusitis, accompanied by concurrent inflammation of oral, nasal, or laryngeal mucous membranes, was of paramount importance. Based on the quality of life questionnaire, used in this study, evident improvement in the masticatory efficiency, speech articulation, and respiration was observed. Particularly good effects were obtained in edentulous patients, in whom implant-prosthetic treatment was possible to apply. Comprehensive and multidisciplinary care of postoperative patients greatly contributes to their better quality of life and facilitates their return to prior living conditions, as well as to occupational and family lives.

Keywords

Head and neck cancer • Maxillary tumors • Obturators • Prosthetic rehabilitation • Respiration • Upper airway inflammation

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1 Introduction

Head and neck cancers make up about 10 % of all malignant neoplasms diagnosed in humans. As much as 90 % of neoplasms are of ectodermal origin and they are mostly squamous cell carcinomas (SCC). They may affect different anatomical structures, such as lips, oral cavity (mucous membranes of bucca, gingiva, hard palate, tongue, or floor of the mouth), oral pharynx (palatal tonsils and soft palate), nasal and laryngeal parts of the pharynx, nasal cavity, paranasal sinuses, larynx, and salivary glands (Gliński et al. 2006). Deformity and functional defects due to neoplastic diseases and their surgical treatment may lead to different stages of dysfunction, comprising basic vital functions such as breathing, eating, or speaking. This applies in particular to mutilating surgery.

The best treatment choice depends on the patient's general condition, clinical staging of cancer advancement, cancer site, and a histopathologic image. Surgery, radiotherapy, and chemotherapy are still the most effective methods for treating squamous cell carcinoma of the head and neck. It should be emphasized that patients under treatment benefit mostly from multidisciplinary team management. The team should include physicians who represent different specialisations such as oncologic surgery, laryngology, maxillofacial surgery, radiotherapy, and prosthodontics. The efficacy of anticancer therapy is still growing, which allows for a significant increase in the cure rate or prolongs the interval of disease remission. However, the applied treatment often contributes to numerous morphological and functional disturbances, which greatly affect the rehabilitation of patients and their quality of life.

Morphological disturbances in the maxillofacial region entail functional disorders in the entire stomatognathic system, as well as in the upper respiratory and alimentary systems The intensity and nature of disorders depend on the site and extent of a defect, and deformity of surrounding tissues. In the midface, when the postoperative defect results from the resection of maxillary bone, the continuity of the dental arch and alveolar ridge is broken and parts of hard and soft palates, zygomatic bone and bottom of eye socket, frequently with all its content (enucleation), are lost. In consequence of surgical treatment, a connection between oral, nasal, and sinus cavities is formed. Oral and nasal connection implicates the development of serious disturbances of basic functions of the stomatognathic system: breathing, eating, and speaking. Eating is considerably impeded by the penetration of gastric contents and liquids into nasal cavity, sinuses, and throat. This is followed by decreased elasticity and mobility of buccal and labial orbicular muscles, reduced mouth opening, and dried mucous membrane (xerostomia) of the upper airways. That, in turn, leads to chronic inflammation of oral and nasal mucous membranes, characterized by a recurrent nature and difficult to treat (Rolski et al. 2007; Gliński et al. 2006).

Ionizing radiation and chemotherapy, despite positive therapeutic effects in cancer treatment, may induce a variety of adverse side effects, as well as morphological and physiological complications in hard and soft tissues of the head and neck region, which substantially impedes prosthetic treatment (Sonis and Fey 2002). Radiotherapy is frequently responsible for mucous inflammation of the oral cavity, tongue, and throat (mucositis), bleeding, pain, or changes of taste. It may also cause inflammation and bone necrosis (Rayatt et al. 2007; Rubinstein et al. 2004; Sonis and Fey 2002).

Serious disorders of salivary gland secretion (xerostomia) due to irreversible changes in glandular tissues, manifested by fibrosis and atrophies, are observed in irradiated patients. Changes in oral biocoenosis following both radiotherapy and chemotherapy, the loss of salivary buffering capacity and low level of immunoglobulins contribute to the development of prosthetic stomatopathy, frequently complicated by fungal infection and secondary bacterial infections, which impairs respiratory functions. In this group of patients fungal infections (candidiasis) are difficult to cure, taking account of

their frequent recurrences and the need to intensify treatment by increasing anti-fungal drug doses and extending treatment time (Szyszkowska et al. 2011; Denis et al. 2004; Rubinstein et al. 2004).

Morphological and functional disorders caused by surgical and supportive treatments exert an adverse effect on the upper airways. In addition to the implementation of the prosthetic rehabilitation process in post-surgical patients, the role of prosthodontists is to use procedures leading to the restoration of morphological and functional defects, to protect, in cooperation with laryngologists, against inflammation of upper airways and provide treatment if necessary, and to improve patients' quality of life.

2 Methods

The study was conducted in accordance with the Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association Declaration of Helsinki.

In the years 1996–2010, prosthetic rehabilitation was accomplished in 494 (219 women and 275 men) patients referred to the Department of Prosthodontics, Faculty of Medicine and Dentistry, Warsaw Medical University in Warsaw, Poland. The patients had previously been subjected to tumor ablative surgery in the head and neck region. Almost half (226; 46 %) of patients under prosthetic rehabilitation underwent neoplasm surgery in the midface, including maxillary bone defects in 208 (92 %) patients. In 14 patients, post-operative maxillary defects were rebuilt by inserting dental implants. In the remaining 194 (86 %) patients, maxillary obturator prostheses were used.

In prosthetic rehabilitation three models of treatment: immediate, early, and long-term, were applied in patients after surgical treatment of maxillary neoplasms. These terms define a time-lag between surgery and the beginning of prosthetic rehabilitation. In immediate treatment, prosthetic restoration was provided directly after surgery, with the patient still on the operating table. Early treatment was performed on patients with maxillofacial surgery as a next phase of prosthetic rehabilitation after an initial period of healing of the post-operative wound (3–4 months after surgery), with the use of removable partial or complete denture with obturators. In non-extensive maxillary defects closed obturators (covered bulb obturators) and in large defects open obturators (hollow bulb obturators) were produced. The long-term treatment involved the choice of post-operative prosthetic restoration that defined the condition of the prosthetic base, localization and extent of deformation, tissue loss and its localization, and the number and condition of retained natural teeth. Current prosthetic rehabilitation offers a wide range of prosthetic constructions, which can be applied in individual clinical cases in patients after midface surgery. Different types of prostheses (removable, complete and partial, fixed, or modified) were used, depending on individual conditions (Oh and Roumanas 2006).

3 Results and Discussion

In patients after maxillary cancer resection, post-operative prostheses with obturators (depending on the number, quality, and distribution of teeth) integrated with the plate of removable, partial or complete denture were used. Of all the treated patients, edentulous patients formed the largest group. Depending on the type and extent of post-operative defects and their localization, complete dentures with obturators of different kind, size and shape, close or open, were produced for this group of patients.

The quality of life (QOL) assessment is the most telling measurement tool used to assess the efficacy of prosthetic rehabilitation in postoperative patients. It provides a comprehensive overview of complex medical problems of physical, mental, and environmental health. There are two possible approaches to the assessment of quality of life, objective – carried out with the participation of medical personnel and subjective – reflecting the patient's feelings. The latter provides the ground for constructing modern

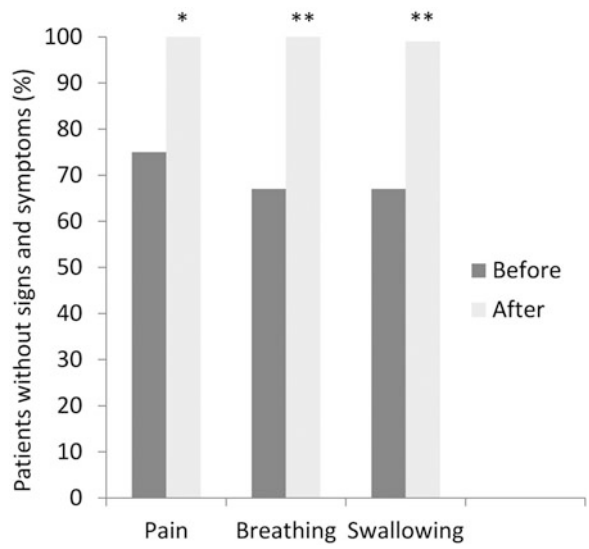

Fig. 1 General assessment of chosen quality of life parameters before and after prosthetic rehabilitation (*p = 0.004; **p = 0.0001; Wilcoxon's test)

instruments, such as questionnaires to assess the quality of life (Weymuller et al. 2001).

The survey carried out in 103 patients, using the University of Washington Quality of Life Revised Version 4 (UW-QOL-R4), which provides basic quality of life data on the physical, functional, and emotional quality of life, and is designed specifically for the population of patients diagnosed with head and neck cancer (Weymuller et al. 2001; Deleyiannis et al. 1997; Hassan and Weymuller 1993). We used the questionnaire to assess the following domains: pain sensation, perception of the patient's own appearance, ability to swallow and breath, mastication efficiency, speech comprehension, sense of taste, and salivation. Scoring is scaled from 0 (worst) to 100 (best). The questionnaire also comprised data on the neoplastic disease, applied surgical treatment, supportive treatment, site and type of the postoperative defect, and also information on prosthetic rehabilitation and the time of adaptation to postoperative prosthetic restoration. The survey was carried out before and after the prosthetic rehabilitation applied to check on its effects. A relation between the type of prosthetic restoration and the patient's evaluation of QOL was evaluated. Wilcoxon's test and Pearson's chi-squared test were used to compare the results. A p-value <0.05 was used to define statistically significant differences. A commercial Statistica ver. 8 package was employed for all statistical elaborations.

In the group of patients with postoperative defects in the midface, impaired speech, eating, and breathing were the most frequent postoperative disorders. The assessment of an influence on QOL parameters of prosthetic treatment revealed a significant lessening of pain sensation ($p = 0.004$) and improvements in the ability to swallow and breathe ($p = 0.0001$) (Fig. 1).

Concerning the assessment of quality of life features, we found that the type of postoperative prosthetic restoration, notably dentures with clasps and precision attachment as opposed to complete dentures, exerted a positive influence on mastication and respiratory efficiency (Fig. 2).

Assessing the efficacy of prosthetic treatment of different midface postoperative defects, the satisfactory results and fast adaptation to postoperative prosthetic restorations were observed in the patients after partial maxillary resection. This applied to both edentulous oral cavity and partial loss of teeth, as well as to a complete resection of one of the maxillas with retained partial denture. In those cases prosthetic restorations greatly contributed to the restoration of the patients' impaired functions. An example of complete upper denture used for prosthetic restoration in the edentulous patient is shown in Fig. 3.

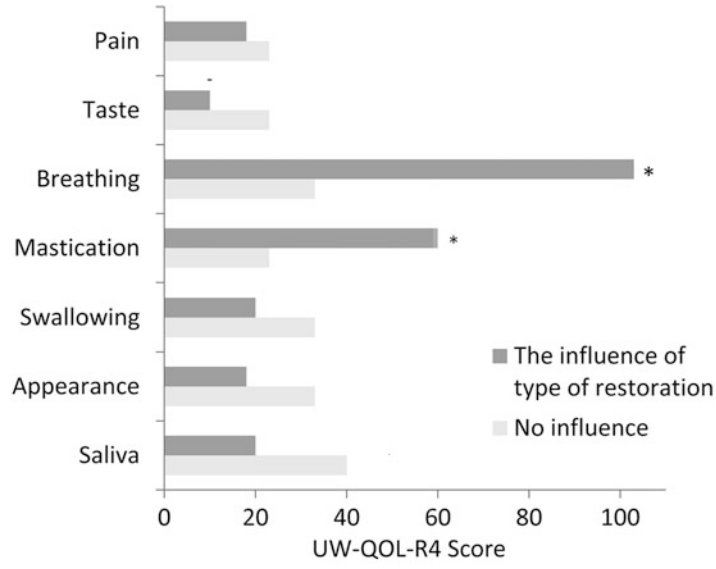

Fig. 2 Assessment of quality of life features with UW-QOL-R4 – University of Washington Quality of Life Revised Version 4 – before and after postoperative prosthetic rehabilitation using dentures with clasps and precision attachment; (*$p < 0.05$; chi-squared test)

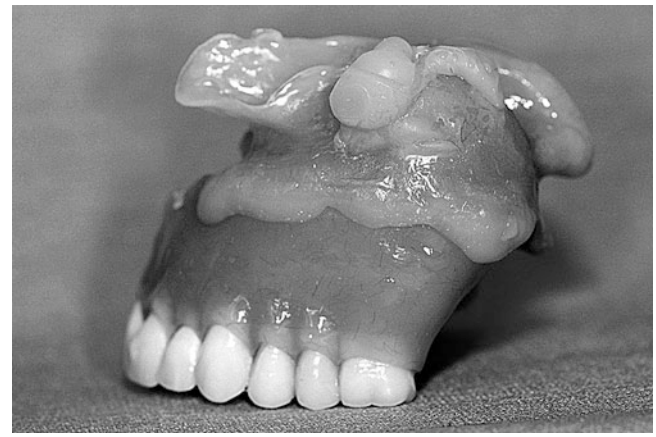

Fig. 3 Postoperative prosthetic restoration –complete upper denture with an obturator relined with soft silicone material

The condition for prosthetic treatment was found to be more difficult in edentulous patients after total resection of the maxilla or its resection extending beyond the midline of the palate. The conditions were even worse in the case of atrophied alveolar ridges, the presence of postoperative scars, or a hollow vestibule of the oral cavity. Large defects, mucous membrane mobility, lack of retention, and the need to relieve sensitive sites of the prosthetic base impeded the satisfactory stabilization of postoperative prosthetic restorations. Moreover, dislocation of prostheses during chewing and liquids penetrating into nasal cavity considerably distorted the harmony of the stomatognathic system. Application of implanto-prosthetic treatment and relining the prosthesis with elastic compounds greatly improved the retention and stabilization of postoperative prosthetic restorations (Fig. 4).

4 Synopsis

- Difficulties in prosthetic rehabilitation of patients after surgical removal of cancer in the head and neck regions are closely related to the outcome of surgery and supportive treatment.

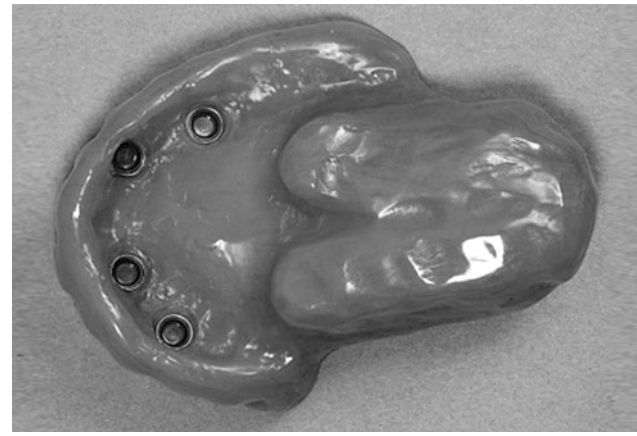

Fig. 4 Postoperative upper complete overdenture integrated with obturator supported by osseointegrated implants

These difficulties impair health-related quality of life of patients, which is reflected in the results of surveys designed to this end.

- Closure of postoperative palatal defects with obturator prostheses improves respiration.
- Involvement of the prosthodontist is an element of multidisciplinary team management representing different dental and medical specialties. Cooperation with laryngologists concerning aspects of chronic paranasal sinusitis with concomitant inflammation of oral and nasopharyngeal mucous membranes is of paramount importance.
- Improvements in patients' quality of life evidenced in this study and clinical observations indicate that a comprehensive and multidisciplinary care of postoperative patients facilitates their return to prior living standards, as well as to occupational and family lives.

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

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Respiratory Toxicity of Dimethyl Sulfoxide

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Abstract

Dimethyl sulfoxide (DMSO) is one of the most commonly used solvents for hydrophobic substances in biological experiments. In addition, the compound exhibits a plethora of bioactivities, which makes it of potential pharmacological use of its own. The influence on respiration, and thus on arterial blood oxygenation, of DMSO is unclear, contentious, and an area of limited study. Thus, in the present investigation we set out to determine the influence on lung ventilation of cumulated doses of DMSO in the amount of 0.5, 1.5, 3.5, 7.5, and 15.5 g/kg; each dose given intraperitoneally at 1 h interval in conscious mice. Ventilation and its responses to 7 % hypoxia (N_2 balanced) were recorded in a whole body plethsymograph. We demonstrate a dose-dependent inhibitory effect of DMSO on lung ventilation and its hypoxic responsiveness, driven mostly by changes in the tidal component. The maximum safe dose of DMSO devoid of meaningful consequences for respiratory function was 3.5 g/kg. The dose of 7.5 g/kg of DMSO significantly dampened respiration, with yet well preserved hyperventilatory response to hypoxia. The highest dose of 15.5 g/kg severely impaired ventilation and its responses. The study delineates the safety profile of DMSO regarding the respiratory function

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which is essential for maintaining proper tissue oxygenation. Caution should be exercised concerning dose concentration of DMSO.

Keywords
Dimethyl sulfoxide • Hypoxia • Mice • Neural function • Respiration • Solvent • Toxicity

1 Introduction

Dimethyl sulfoxide (DMSO) is an amphoteric compound able to permeate through biological barriers, which has since long been used as a solvent and carrier for hydrophobic substances in neurobiological experiments. There is a consistent impression that DMSO is the archetype solvent of low, if not negligible, toxicity, which is supported by extensive veterinary experience (Kelava et al. 2011; Brayton 1986). Nonetheless, toxicological profile of DMSO is an area of limited understanding and not yet fully explored. DMSO exhibits biological activities which quite often assume opposing face. The compound is neuroprotective in traumatic brain (Di Giorgio et al. 2008) and spinal cord injury (Goodnough et al. 1980), or in brain ischemic infarction (Bardutzky et al. 2005; Shimizu et al. 1997). On the other hand, DMSO may be neurotoxic as it facilitates brain edema, disrupting the brain-blood barrier integrity in the middle cerebral artery occlusion model in the rat (Kleindienst et al. 2006). DMSO-related neurotoxicity associated with ischemic brain infarction has been reported following peripheral blood stem cell transplantation in patients receiving high dose chemotherapy (Windrum and Morris 2003).

Likewise, protective effects of DMSO on myocardium have been reported, liable to result from the compound's antioxidant, anti-inflammatory, and Na^+ channel blocking actions (Bini et al. 2008; Lu and Mattson 2001; Regoli and Winston 1999). In opposition to this, DMSO is implicated in the development of heart arrhythmias and conduction disorders due its hypervagal activity or histamine release in patients during peripheral blood stem cell infusion (Ferruci et al. 2000; Keung et al. 1994).

Among a plethora of bioactivities of DMSO, its effects on respiration have grossly escaped attention. The reports are scarce and concern respiratory depression, randomly unraveled as an adverse effect of DMSO-cryopreserved hematopoietic stem cell transplantation (Benekli et al. 2000). It is unclear, however, whether the respiratory depressive effects could be ascribed to DMSO *per se* or are secondary to interaction with analgesics routinely used in such cases. Nonetheless, DMSO promotes mitochondrial damage and apoptosis of astrocytes (Yuan et al. 2014), which are operative in central respiratory regulation (Angelova et al. 2015; Okada et al. 2012). It also disrupts myelin sheath and reduces velocity of impulse conduction in peripheral nerves (Cavalletti et al. 2000), blocks propagation of action potentials (Larsen et al. 1996), and stimulates chemical synapse at neuromuscular junctions (Cherki-Vakil and Meiri 1991); all of which is liable to participate in shaping brain motor outputs. Respiration is a fine motor act driven by signals generated in the brainstem and running down to the chest respiratory muscle pump, which, in turn, drives lung expansion. Lung ventilation is thus the ultimate measure of respiratory efficiency. In the present study we seek to determine the effects of increasing doses of DMSO on lung ventilation in the mouse.

2 Methods

2.1 Animals

Experiments were performed in seven male C57BL/6 mice (aged 7.1 ± 1.8 weeks, weighing 22.3 ± 3.6 g) housed at a controlled temperature (25 °C), and exposed to a daily 12:12-h light-

dark cycle. The experimental protocols were in accordance with the Guiding Principles for the Care and Use of Animals of the Physiological Society of Japan, and were approved by the Animal Care and Use Committee of the National Hospital Organization Murayama Medical Center in Musashimurayama, Tokyo.

2.2 DMSO Injections

Cumulative DMSO injections were made i.p. at approximately 1 h intervals in the following experimental sequence.

1. Saline 1.82 mL/kg
2. DMSO 0.46 mL/kg + Saline 1.36 mL/kg (DMSO dose: 0.5 g/kg)
3. DMSO 0.91 mL/kg + Saline 0.91 mL/kg (DMSO cumulative dose: 1.5 g/kg)
4. DMSO 1.82 mL/kg (cumulative dose: 3.5 g/kg)
5. DMSO 3.64 mL/kg (cumulative dose: 7.5 g/kg)
6. DMSO 7.28 mL/kg (cumulative dose: 15.5 g/kg)

DMSO is a long acting compound. The metabolites, dimethylsulfone and dimethyl sulfide, are mostly excreted in urine and feces. A prolonged excretion taking hours after a single dose, irrespective of DMSO concentration (DMSO 2007), made it optimal to accumulate sequential doses delivered every hour and to investigate each cumulative dose as a separate dose concentration.

2.3 Respiratory Measurement

A whole-body plethysmograph (PLY 310; EMMS, Bordon, UK) consisting of a recording chamber and a reference chamber was used to assess respiratory function (Fig. 1). Conscious unrestricted animals were placed in the recording chamber 30 min after each DMSO injection. The plethysmograph was placed in a transparent acrylic box (20 × 20 × 20 cm) into which either room air (control) or hypoxic gas mixture was delivered at a rate of 2.0 L/min. The air in the recording chamber was continuously suctioned with a flow generator (Air 110; EMMS, Bordon, UK) at a constant rate of 1.0 L/min to ventilate the chamber. The pressure difference between the two chambers, which is directly proportional to respiratory flow, was measured with a differential pressure transducer (TPF 100; EMMS, Bordon, UK).

The mice were first exposed to room air. After the acclimatization period, the pressure difference was measured for 1 min (baseline phase). The N_2 gas was delivered until the oxygen concentration in the chamber reached 7 %, which took about 1 min, and the concentration was further supplied for 2 min. Oxygen concentration was monitored with a rapidly responding O_2

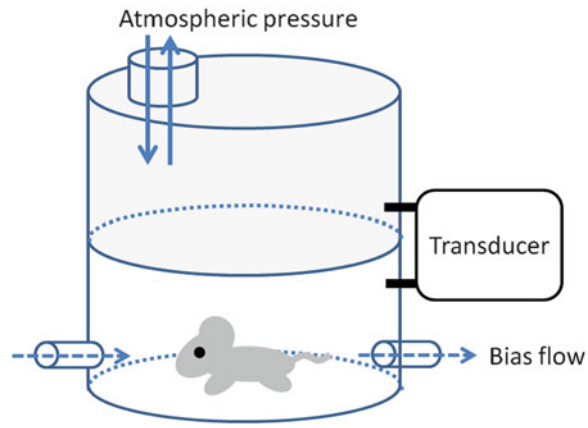

Fig. 1 Scheme of whole body mice plethysmography

analyzer (Respina IH 26, San-ei, Tokyo, Japan). During the hypoxic period, continuous 1 min measurements were taken twice (phases: hypoxia 1 and hypoxia 2). Then, the gas was switched back to room air and 1 min measurements were taken twice again (phases: recovery 1 and recovery 2). The hypoxic ventilatory responses were poikilocapnic.

2.4 Data Elaboration

All data were given as means ± SE. Respiratory flow was calculated from the pressure difference using eDaq software (EMMS, Bordon, UK). Instantaneous minute ventilation (V_E; mL/g body mass/min) for each breathing cycle was calculated as a product of the corresponding tidal volume (V_T; μL/g), extracted from the integration of the flow data, and breathing frequency (*f*; breaths/min). All variables were then averaged for 1 min. Recording intervals during which the mouse exhibited unusual anxiety, such as sniffing, grooming, or licking, were excluded from calculations. The mean values of V_E, V_T, and *f* were submitted to two-way within-subject ANOVA; with six experimental conditions and five oxygen concentration phases (baseline, hypoxia 1 and 2, and recovery 1 and 2). The number of seven mice used in the study was insufficient to calculate Mauchly's W concerning sphericity assumptions for the interaction of experimental condition and oxygen phase. To resolve this problem, Greenhouse-Geisser's correction was used under the presupposition that the sphericity assumption was violated. The *post-hoc* Bonferroni correction was applied for multiple comparisons when significant main effects or interactions were obtained. To specifically evaluate the hypoxic ventilatory response in each DMSO condition, a difference in V_E from baseline to the average value of a 2-min hypoxic phase was compared with that in the saline condition, using Dunnett's *t*-test. The significance level was set at $p < 0.05$. The signal processing was performed using MATLAB 2007b (MathWorks; Natick, MA) and statistical analyses were performed using SPSS 15.0 (IBM; Chicago, IL).

3 Results

Representative original recordings of respiratory flow at baseline, hypoxia, and recovery in the saline and four DMSO conditions (1.5, 3.5, 7.5, and 7.5 g/kg) are shown in Fig. 2. Hypoxic hyperventilation was mostly driven by increases in the tidal component, with a minor contribution of breathing frequency. Tidal ventilation was also the first to be affected by DMSO at a dose of 7.5 g/kg, despite still grossly unchanged frequency. Ventilation was severely impaired at a DMSO dose of 15.5 g/kg. Afflicting the tidal

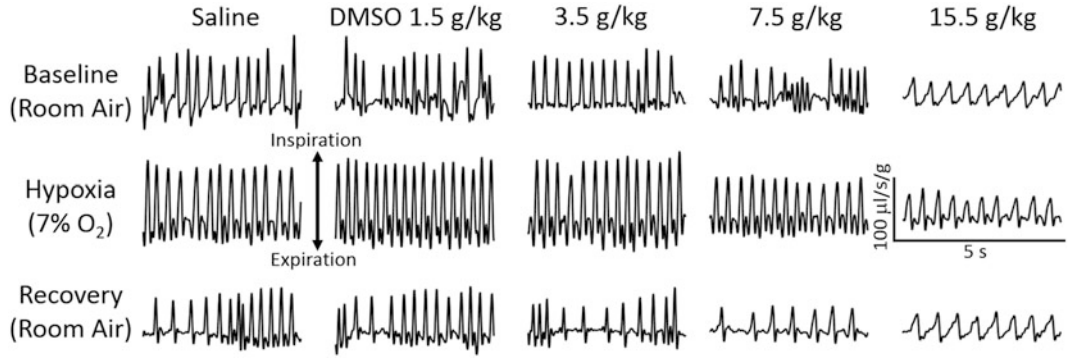

Fig. 2 Raw recordings of respiratory flow exemplifying the influence of increasing doses of DMSO as compared with the control saline condition. Distinct depression of respiration began at 7.5 g/kg and turned into outright suppression of respiration at 15.5 g/kg DMSO

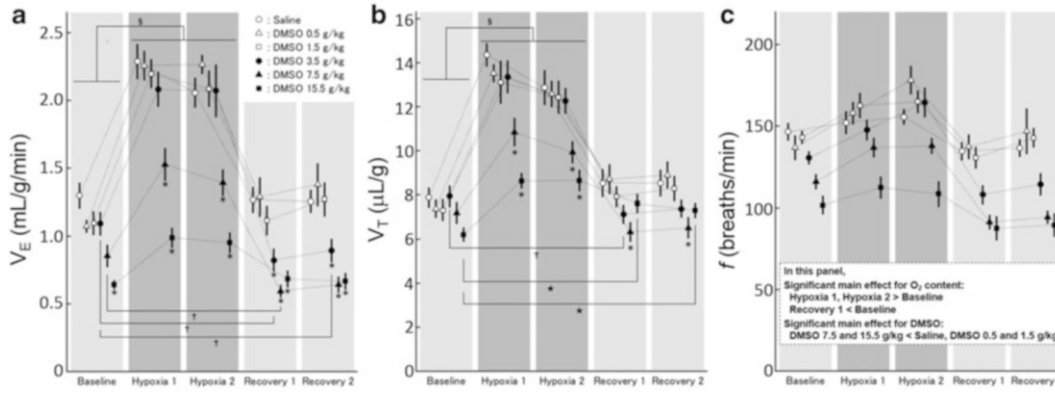

Fig. 3 Minute ventilation (V_E), volume (V_T) and frequency (f) components at normoxic baseline, and during hypoxia 1 ~ 2 and recovery 1 ~ 2 phases. § significant increase in hypoxic phase against baseline, † significant decrease in recovery phase against baseline, ★ significant increase in recovery phase against baseline, * significant decrease against saline condition in the same phase

component is suggestive of central effects on neuronal mechanisms generating inspiratory drive in the brainstem (Izumizaki et al. 2004) rather than on chemosensing mechanisms in the carotid body, which mostly influence breathing frequency (Coles et al. 2002).

From these recordings the mean values of V_E, V_T, and f were tallied at sequential DMSO doses and compared with the corresponding baseline levels in the control saline condition (Fig. 3). The hypoxic ventilatory response showed a trace of a fall-off in the second minute of exposure, which was more accentuated, albeit insignificantly so, in V_T (Fig. 3b). Compensatory increases in f up to a DMSO dose of 3.5 g/kg enabled to maintain the stimulatory level of V_E (Fig. 3c). The two highest doses of DMSO of 7.5 and 15.5 g/kg caused a significant dampening of the hypoxic V_E in both minutes of recording, which reflected similar changes in V_T. The 7.5 g/kg dose of DMSO, although significantly inhibitory for the hypoxic V_E, still allowed ventilation to about double from its baseline level and reach a reasonable 1.5 mL/g/min range; the level unattainable after the 15.5 g/kg DMSO. Both these doses also decreased baseline normoxic ventilation. Interestingly, the most sensitive to the depressant action of DMSO appeared the recovery from hypoxia. A difficulty to recover was noted already at the 3.5 g/kg DMSO, a dose that yet caused no appreciable effect on either normoxic or hypoxic ventilation. Recovery ventilation hardly hovered around the baseline level at higher DMSO doses.

Calculations revealed significant main effects on V_E of experimental condition ($F_{5,\ 30} = 67.3$, $p < 0.01$), oxygen concentration ($F_{2.4,\ 14.2} = 376.9$, $p < 0.01$), and the interaction between the two ($F_{4.33,\ 26.0} = 9.6$, $p < 0.01$) (two-way ANOVA). The *post-hoc* tests carried out for the interaction showed significant increases in V_E from baseline room air to hypoxia at each DMSO dose. At the 7.5 and 15.5 g/kg DMSO, there were significant decreases in the hypoxic and recovery V_E levels compared with the saline control (Fig. 3a).

We also compared the magnitude of augmentation of minute ventilation (ΔV_E) from the baseline to hypoxic level. The ΔV_E amounted to 0.87 ± 0.09 mL/g/min in saline and 0.98 ± 0.09 mL/g/min at the 3.5 g/kg DMSO, but it decreased to 0.60 ± 0.04 and 0.33 ± 0.05 mL/g/min at the 7.5 and 15.5 g/kg DMSO doses, respectively.

Changes in V_E were in harmony with those in V_T. Likewise, there were main effects on V_T of experimental condition ($F_{5,\ 30} = 31.3$, $p < 0.01$), oxygen concentration ($F_{4,\ 24} = 347.0$, $p < 0.01$), and the interaction between the two $F_{3.7,\ 22.3} = 11.7$, $p < 0.01$. The *post-hoc* tests carried

out for the interaction showed significant increases in V_T from baseline room air to hypoxia at each DMSO dose. At the 7.5 and 15.5 g/kg DMSO, there were significant decreases in the hypoxic V_T levels, and at the 15.5 g/kg DMSO also in the recovery V_T level, compared with the saline control (Fig. 3b).

The concurrent changes in *f* were less remarkable. There were main effects on *f* of experimental condition ($F_{5,\ 30} = 28.8$, $p < 0.01$) and oxygen concentration ($F_{4,\ 24} = 48.3$, $p < 0.01$), but no significant interaction between the two. The *f* increased significantly in hypoxic phases and decreased in recovery 1 compared with the baseline level. The mean *f* at the 7.5 and 15.5 g/kg DMSO doses were significantly smaller than those in saline, and 0.5 and 1.5 g/kg DMSO conditions.

4 Discussion

In the present study we investigated whether DMSO, a widely used laboratory solvent for lipid-soluble substances, might affect respiratory function. The main finding was a dose-dependent suppression of lung ventilation with DMSO in the conscious mouse. The study delineated a functional safety profile of DMSO regarding respiration, in which several dose thresholds of increasing toxicity can be recognized. DMSO at a dose of up to 1.5 g/kg did not affect respiration. The dose of 3.5 g/kg, albeit not changing resting ventilation and its stimulatory responses to hypoxia, caused an appreciable undershoot in the recovery period, making it attainable for lung ventilation to revert toward the baseline pre-hypoxic level. Interestingly, the first subtle sign of ventilatory deficiency appeared not in the stimulatory augmentation, but rather unexpectedly in recovery from the strain put on the system. That finding points attention to the recovery phase, often in neglect, as a sensitive functional stage.

Respiratory insufficiency became distinct at the higher doses of DMSO of 7.5 and 15.5 g/kg throughout all phases studied, i.e., normoxia, hypoxia, and recovery. The former dose, despite the inhibition of ventilation and its responses, failed to abrogate ventilatory responsiveness, as ventilation about doubled in hypoxia. The latter dose left little ability to respond to hypoxia and caused across-the-board ventilatory dampening. The DMSO dose of 15.5 g/kg, however, borders the single intraperitoneal dose LD50, which is variably reported in a range of 14.7–17.0 or 13.4–15.5 g/kg for the mouse (DMSO 2007; Caujolle et al. 1964; Farrant 1964), although lower LD50 doses in a range of 7.0 g/kg have also been reported (Bartsch et al. 1976). Such high doses have not actually been reported for use as a vehicle in the literature.

Lung ventilation is a sensitive element of a compound's toxicity as it underlines arterial blood oxygen content, and therefore tissue oxygenation, being crucial for maintaining body homeostasis. Among a multitude of bioactivities exhibited by DMSO regarding the central nervous, cardiovascular, renal, gastrointestinal, and other systems (Kelava et al. 2011), the reports on respiratory toxicity of DMSO are meager and the issue is contentious. Respiratory depression has been reported as a side effect in connection with transplantation of cryoprotected with DMSO autologous hematopoietic stem cells in young cancer patients (Caselli et al. 2009). The individual doses of DMSO, routinely used for cryoprotection, were in a range of 0.18, 0.44, and 1.30 g/kg. The patients were on chemotherapy associated with heavy analgesic medication with morphine. Morphine, as are also other opiates, is a selective respiratory depressant (Budzińska et al. 1985). DMSO, an analgesic in its own right (Hoang et al. 2011; Haigler and Spring 1981), potentiates anti-nociceptive effects of morphine (Fossum et al. 2008). Therefore, the reported respiratory depression is liable to represent an interaction of drugs rather than a true DMSO-dependent hypoventilation. There are also reports of no essential effects of 6.5 g/kg intraperitoneal DMSO on respiratory responses to endotoxin in awake rats (Brackett et al. 1991) or 1 g/kg of 10 % intravenous DMSO in awake horses on cardiopulmonary function (Lin et al. 2004). Apparently, diversity of findings has to do with pharmacological interactions,

and a spate of experimental settings used in studies.

Recently, research interest in DMSO goes beyond its being a tool conducive to other substances dissolution and delivery. DMSO has attracted continuing attention as a potential clinical pharmacotherapeutic due to its neuroprotective, antioxidant, analgesic, and anti-inflammatory properties (Kelava et al. 2011; Harter 1983). For now, DMSO is accepted only as intravesical therapy in interstitial cystitis (Desrosiers and Garely 2015). However, search for other potential medicinal uses continues, which warrants careful consideration of adverse effects, particularly in the areas of still limited understanding such as the effect of DMSO on respiratory regulation.

To wrap it up, we demonstrate a dose-dependent inhibitory effect of DMSO on lung ventilation and its hypoxic responsiveness. The recommendation from the present study is to consider a DMSO dose of 3.5 g/kg as the upper limit of null or hardly meaningful consequences for respiratory function. The uncrossable limit ought to be 7.5 g/kg of DMSO, a dose which dampens respiration but not respiratory responsiveness. Caution should be exercised when DMSO is used as a solvent in bioexperiments.

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Conflicts of Interest The authors report no conflicts of interest in relation to this article. The study had no commercial funding.

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Index