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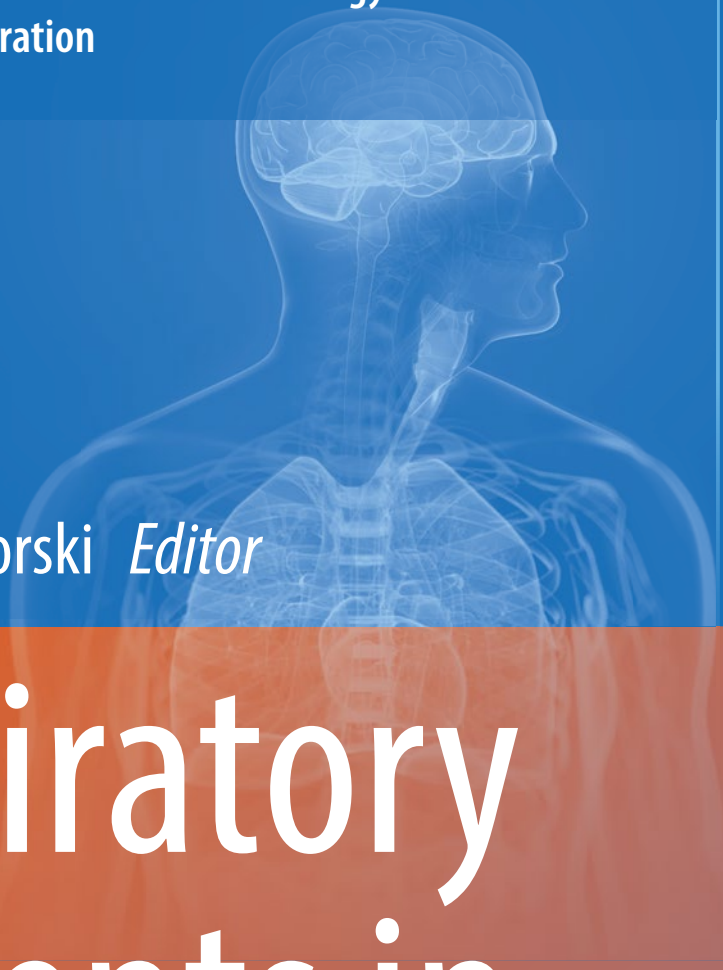

Mieczyslaw Pokorski *Editor*

Respiratory Ailments in Context

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Mieczyslaw Pokorski
Editor

Respiratory Ailments in Context

Springer

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Preface

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

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

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

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

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

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

Mieczyslaw Pokorski

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Methodological Implications and Repeatability of Nasal Nitric Oxide: Relevance for Challenge Studies

Frank Hoffmeyer, K. Sucker, H. Berresheim, C. Monsé, B. Jettkant, A. Beine, M. Raulf, T. Brüning, and J. Bünger

Abstract

There is an interest in assessing changes in nasal NO (nNO) levels as an effect marker of upper airways. In this study, we examined methodologic influences on short and long term repeatability of nNO levels assessed by a portable electrochemical analyzer. Nine atopic and eighteen healthy subjects were exposed for 4 h to ethyl acrylate concentration of 0.05 ppm (sham) and mean concentrations of 5 ppm (either constant 5 ppm or variable 0 to 10 ppm). Sampling of nNO was performed by using passive aspiration during both breath-holding (634 ppb) or calm tidal breathing (364 ppb, $p < 0.0001$). The intra-session (between-session) repeatability in terms of coefficient of variation was 16.4% (18.5%) using the tidal-breathing and 8.6% (13.0%) using the breath-holding method, respectively. Atopic subjects demonstrated a significant increase in nNO (breath-holding mean 16%, tidal-breathing mean 32%) after applying a constant ethyl acrylate concentration (5 ppm). Our findings suggest that the less elaborate tidal-breathing method might be sufficient to detect significant changes at a group level. Given a lower coefficient of variation of breath-holding we assume there is an advantage of that approach at an individual level. Further research is needed to validate the usefulness of nNO in the evaluation of irritative, non-allergic responses.

Keywords

Atopic subjects · Breath holding · Chemosensory challenge · Ethyl acrylate · Inflammation · Methodological approach · Nasal nitric oxide · Tidal breathing · Upper airway

1 Introduction

Sensations of odor and upper airway irritation are cited health effect in indoor air and occupational environments and have gained relevance for the setting of exposure limits (Brüning et al. 2014). Besides symptom complains, response to a chemosensory irritant can also be evaluated by examining local signs of nasal mucosal irritation (Arts et al. 2006). Concerning ethyl acrylate, a trigeminal intranasal perceptions and signs of nose irritation could be provoked by challenge with a concentration of 5 ppm (Hoffmeyer et al. 2016). Objective measures of upper airway irritation include rhinomanometry or acoustic

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rhinometry to measure functional changes such as irritation-induced congestion, measuring the volume of mucus secretion or the presence of inflammatory mediators in nasal secretion (Raulf et al. 2017; Dalton 2003). The evaluation of mediators in different matrices is a promising way to get information on inflammation at target level in a noninvasive manner (Quirce et al. 2010).

Concerning the lower airways, NO in exhaled breath (FeNO) is a validated noninvasive marker of inflammation, especially when associated with the activation of eosinophils (Dweik et al. 2011). NO is also produced in the nasal epithelium or can be a result of diffusion from communicating cavities, i.e., paranasal sinuses (Maniscalco et al. 2016). There is a continuous and high basal activity of NO synthase resulting in a 100-fold higher nasal NO (nNO) compared to NO produced in the lower respiratory tract (Lundberg et al. 1995). A variation in inducible NO synthase expressed under basal conditions in the human nasal mucosa has been reported (Furukawa et al. 1996). Anatomical means, such as sinus ostiae diameter and volume, may influence the NO output (Palm et al. 2000).

Metabolic or dietary factors, ambient NO concentration, and human biorhythm have been all suggested to be responsible for the variability of nNO level (Gehring et al. 2009). A diurnal variation in nNO with low levels in the morning, a plateau during the day, and decreasing levels in the evening has been reported (Dressel et al. 2008; Palm et al. 2000). NO in the upper respiratory tract associates with the patient's condition. It often increases in allergic rhinitis and decreases after treatment with nasal glucocorticoids (Kharitonov et al. 1997). A dramatic decrease in nNO has been shown in primary ciliary dyskinesia (Wodehouse et al. 2003). So far, with the exception of ciliary disorders, detection of nNO is not a routine diagnostic method (Antosova et al. 2017).

Nevertheless, there is an interest in assessing changes in nNO as an effect marker for inflammatory processes in the upper airways either in short term after a specific challenge or in longitudinal studies. Low variability and good repeatability in these settings are critical in distinguishing changes due to biological responses from methodological variations. Several issues related to the measurement and reproducibility of nNO have been identified and methodological recommendations have been developed (Horváth et al. 2017). Besides a setup for active single-breath nasal exhalation (FnNO) similar to orally exhaled FeNO (Palm et al. 2000), consensus recommendations have suggested methods of passive aspiration *via* one nostril (ATS/ERS 2005). Using nasal aspiration, the highest nNO values have been found in the methods with an elevated velum as during breath-holding or while exhaling against a resistance (de Winter-de Groot and van der Ent 2009). A recent suggestion is that calm breathing through the mouth while avoiding any kind of breathing through the nose creates two distinct, independent compartments (oropharynx and nasopharynx) that are not connected to each other (Gelardi et al. 2016).

In this study, we examined methodologic influences on short and long term repeatability of nNO levels assessed by a portable electrochemical analyzer. NO sampling was performed by using passive aspiration during breath-holding and calm breathing. The evaluations were done within the frame of an ethyl acrylate challenge study reported recently (Hoffmeyer et al. 2017). Therefore, the atopic status of the subjects and effects of different challenge patterns on nNO changes were also considered.

2 Methods

The study was approved by a local Ethics Committee of the Ruhr University Bochum and was performed in accordance with the Declaration of Helsinki. All study participants gave written informed consent and received financial compensation for their participation.

2.1 Subjects

Twenty-seven healthy subjects were recruited for the study after a medical history was taken. All

Table 1 Characteristics of subjects

Gender, F/M (n)	15/12
Age (year)	24 (23; 27)
BMI (kg/m^2)	22.0 (19.9; 23.9)
sx1 (kU/L)	0.11 (0.10; 1.65)
FEV_1 (%pred$_{GLI}$)	97.7 (91.4; 104.8)
z-score	−0.20 (−0.73; 0.40)
FVC (%pred$_{GLI}$)	101.7 (93.2; 110.1)
z-score	0.15 (−0.54; 0.84)
FEV_1/FVC (%pred$_{GLI}$)	97.0 (95.0; 100.7)
z-score	−0.38 (−0.70; 0.19)

Continuous variables are medians and inter-quartile range (IQR). *BMI* body mass index, *sx1* IgE antibodies to a mixture of ubiquitous allergens (atopy screen), *FEV_1* forced expiratory volume in 1 s, *FVC* forced vital capacity, *GLI* global lung initiative

subjects were healthy never-smokers or had terminated smoking at least half a year before the study and were not taking any medications or nasal decongestants (Table 1). A positive atopic status was assumed in case of specific IgE concentrations to common inhalant allergens ≥0.35 kU/L (sx1 Phadiatop; ThermoFisher Phadia AB; Uppsala, Sweden). No one had an upper or lower airway respiratory disease or infection within 6 weeks prior to the study. No signs of mucosal inflammation or anatomical changes were noted on rhinoscopy. Subjects were informed not to perform any strenuous physical activity within 60 min before the nNO measurements. All asymptomatic subjects serologically identified as being atopic were tested outside their potential allergic risk seasons revealed by prick testing.

2.2 Nasal NO Measurements

The measurements were performed using the nasal application of the hand-held NIOX MINO® system (Circassia, Bad Homburg, Germany) under supervision of the same assistant in accordance with published recommendations (ATS/ERS 2005). NIOX MINO® employs an electrochemical sensor with a measurement range of 5–1700 ppb. Short term repeatability at a flow rate of 5 ml/s was shown to be good with a coefficient of variation of 10% (Marthin and Nielsen 2013).

The subjects were studied in a convenient seated position. The nasal olive application was inserted into one nostril and hold in place by the participant while the contralateral nostril was left open. The aspiration was done at a flow rate of 5 ml/s and automatically stopped after 45 s. Two different aspiration techniques were applied for the measurement. Firstly, subjects performed oral calm breathing during the meausrement aspiration, while avoiding any kind of breathing through the nose. No exhalation resistance was used. Secondly, the measurement was done again during breath-holding while the mouth was closed.

Measurements were done in the frame of a challenge study previously published (Hoffmeyer et al. 2017). Briefly, nNO was measured before (pre) and after (post) challenge with a mean ethyl acrylate concentration of 5 ppm applied in a constant and variable wave-form pattern. The study was randomized and sham-controlled (0.05 ppm ethyl acrylate).

2.3 Statistical Analysis

Coefficient of variation was used to describe the repeatability. It was estimated as a ratio between the within standard deviation (SD) and the mean of individual measurements. The Bland-Altman analysis was performed for comparison of the two methodological approaches and different time points. The effect of nNO was calculated as the percent change after exposure compared to start of exposure [(post-pre)/pre*100%] and reported as ΔnNO. Differences after exposure to 5 ppm were compared with those after sham exposure using a paired *t*-test or Wilcoxon matched-pairs signed-rank test as appropriate, with a significance level of $\alpha = 0.05$. Data were expressed as means ±SD or medians with interquartile range (IQR, 25th; 75th percentile). Data were analyzed and visualized by GraphPad Prism v7.03 for Windows (San Diego, CA).

3 Results

3.1 Comparison of nNO Measurements

In the 27 subjects studied, approved nNO measurements were obtained at all occasions with both methodological approaches. At every session (sham, constant, and variable ethyl acrylate concentration) pre- and post-challenge measurements were performed. Therefore, 162 pairs of values could be used for the methodologic comparison. The assessment of between-session repeatability for each method could be based on 81 (27 triplets) respective pre-challenge results.

Referring to all 162 measurement pairs, the nNO level for tidal-breathing [364 (IQR 259; 497) ppb] was significantly lower than that for the breath-holding (reference) [634 (IQR 499; 771) ppb] ($p < 0.0001$). The Bland-Altman analysis of distance for the two methods revealed a mean nNO difference of 243 ppb (50%). The SD of the difference was 191 ppb (51%) yielding the limits of agreement of −131 ppb (−18%) and 616 ppb (121%). The Bland-Altman plot revealing two subgroups is shown in Fig. 1. One subgroup of subjects demonstrated a small difference between nNO results for the tidal-breathing and breath-holding methods. As shown in Fig. 1, the difference was lower than 15% in 40 comparisons and the methods could be considered equally. Thirty-seven of these equal results for the tidal-breathing and breath-holding methods could be attributed to seven out of the 27 subjects.

3.2 Repeatability of nNO Due to Method Applied

3.2.1 Intra-session Repeatability

The condition with sham exposure was used to examine the intra-session repeatability of nNO, as individual variation ($n = 27$) between baseline and post-challenge measurement. The coefficient of variation was 16.4% (95%CI 11.7–21.1) using the tidal-breathing and 8.6% (95%CI 6.0–11.2) using the breath-holding method (Table 2). In addition, the Bland-Altman analysis revealed a bias of 3.9% and 0.6% between first and second measurement for the tidal-breathing and the breath-holding method, respectively (data not shown). Overall, nNO levels were not different at baseline (9 am) and post-challenge (1 pm).

3.2.2 Between-Session Repeatability

At all three sessions, significantly lower nNO levels for the tidal-breathing compared to the breath- holding method were observed at baseline (for each session $p < 0.0001$). Referring to one particular method, no significant difference in the baseline nNO level could be determined between the three sessions (tidal-breathing method; $p = 0.249$, breath-holding method; $p = 0.452$). The individual between-session repeatability in terms of the coefficient of variation was 13.0% (95%CI 9.4–16.7) for the breath-holding and 18.5% (95%CI 12.4–24.5) for the tidal-breathing method (Table 2).

3.2.3 nNO Levels After Ethyl Acrylate Challenge

The two challenge conditions (constant, variable) resulted in an equivalent total amount of ethyl acrylate delivered during 4 h. The effects were also assessed following the sham exposure. Table 3 summarizes the results for these three exposure conditions and the two measurement modes. No changes in nNO could be observed either after sham or variable ethyl acrylate exposure regardless of the measurement technique. In contrast, nNO increased after applying a constant pattern. Despite differences in the baseline levels, the increase was significant for both measurement modes (breathhold; $p = 0.035$, tidal breathing; $p = 0.030$).

Next, results were stratified by the atopic status using $sx1 \geq 0.35$ kU/L as a cut-off value. Accordingly, nine subjects were classified as atopic. Baseline, pre-exposure nNO did not differ between atopic and non-atopic subjects before sham, variable, or constant ethyl acrylate challenge, regardless of the measurement technique used (data not shown).

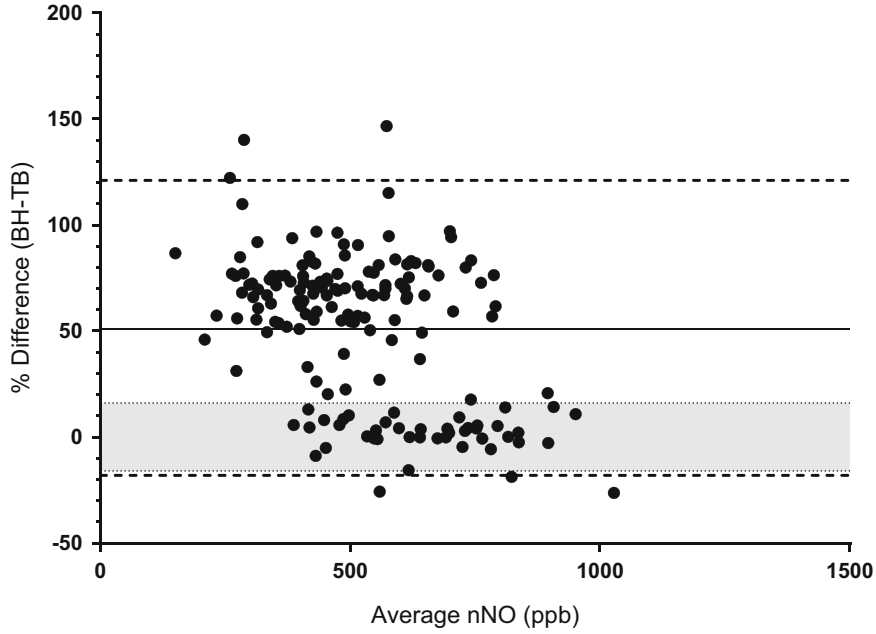

Fig. 1 Bland-Altman plot of agreement between nasal NO (nNO) measured during breath-holding (BH) and tidal-breathing (TB) for all time points (n = 162). The continuous line represents the mean difference and the dashed lines represent the ±2SD for the differences. The shaded area indicates the values that could be considered equalin both methods of measurement

Table 2 Intra-session and between-session repeatability of nNO measurement in 27 subjects fort the two different sampling methods

Repeatability method	Mean nNO (ppb)	*s*	Coefficient of variation (95% CI)
Intra-session			
Breath-holding	654	60	8.6 (6.0–11.2)
Tidal-breathing	411	64	16.4 (11.7–21.1)
Between-session			
Breath-holding	641	84	13.0 (9.4–16.7)
Tidal-breathing	394	71	18.5 (12.4–24.5)

S estimate of the mean pooled within SD, *Coefficient of variation,* estimate of the mean pooled coefficient of variation, *LL* lower limit, *UL* upper limit

Changes in nNO adjusted for the sham condition (net-response) for both methodological approaches are illustrated in Fig. 2. In non-atopic subjects, no significant net-effect on nNO could be observed after either constant or variable ethyl acrylate exposure, regardless of the measurement technique. Atopic subjects demonstrated a significant increase in nNO (breath-holding mean 16%, tidal-breathing method mean 32%) after applying a constant ethyl acrylate concentration (5 ppm). Differences in net-ΔnNO in atopics compared to non-atopics were significant using breath-holding ($p = 0.043$). In case of the tidal-breathing technique, differences due to the atopic status could also be suggested only after applying a constant ethyl acrylate exposure pattern ($p = 0.067$).

Table 3 Changes in nNO (ppb) according to ethyl acrylate challenge condition and measurement mode (n = 27)

	Breath-holding		Tidal-breathing	
Condition	Pre	Post	Pre	Post
0.05 ppm	632	635	335	371
	(499–754)	(521–764)	(241–472)	(280–485)
5.0 ppm	603	**676***	331	**393***
	(447–809)	(460–788)	(234–463)	(276–549)
0 to 10 ppm	600	603	311	371
	(506–756)	(491–749)	(246–466)	(276–522)

Values are medians (25th – 75th percentile); *Statistically significant from pre-exposure at $p < 0.05$; paired *t*-test

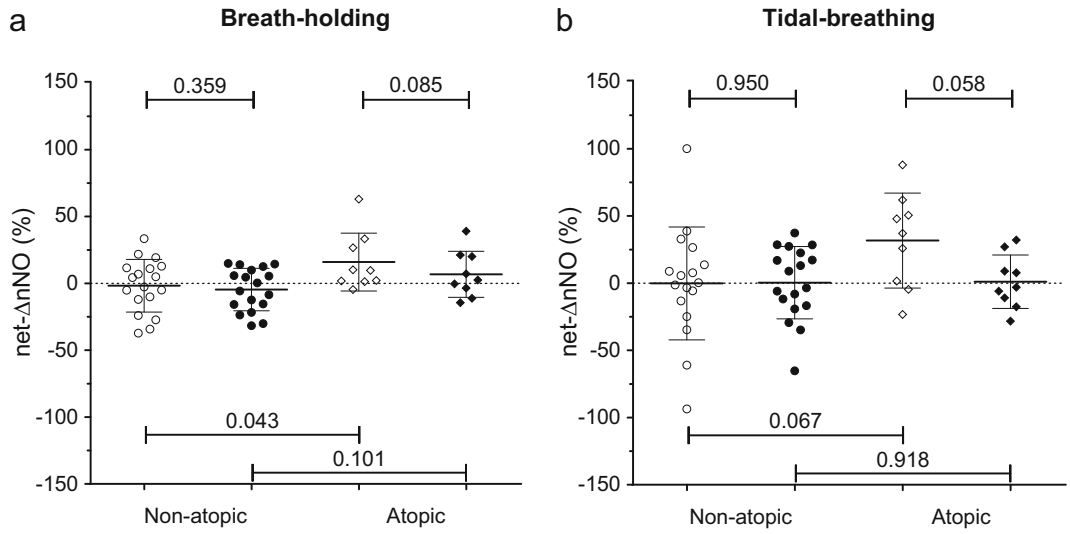

Fig. 2 Net responses of nNO after ethyl acrylate challenge (mean 5 ppm) using the breath-holding (**a**) and tidal-breathing method (**b**). Ethyl acrylate was applied either in a constant (5 ppm, open symbols) or variable pattern (0–10 ppm, solid symbols). Results are adjusted for sham exposure and stratified according to non-atopics (n = 9, circle) and atopics (n = 18, rhombus)

4 Discussion

Reproducibility of clinical tests is an important consideration in research protocols. It is to stress that variability is the result of different influences including analytical impact, technical factors of collection, and intra-subject variability (Antosova et al. 2017). In this study we could demonstrate a better short- and long-term repeatability using the breath-holding than the tidal-breathing technique for nNO measurement. In a challenge setting and referring to comparisons based on groups, the tidal-breathing technique demonstrated sufficient performance in detecting nNO changes.

The actual concentration of NO could be assessed either by chemiluminescence or by electrochemical means. The hand-held electrochemical analyzer NIOX MINO used in this study is a reliable, simple to handle analytic tool that demonstrates the NO values in good agreement with those obtained by stationary chemiluminescence systems (Montella et al. 2011; Maniscalco et al. 2008). Nasal application of the analyzer employs an aspiration time of 45 s, a length shown to be sufficient to obtain measurements at breath-hold within a steady NO plateau (Kharitonov et al. 2005). However, even short interruptions during sampling result in measurement error and require repeated attempts.

Reservoir and continuous techniques for nNO measurement can be distinguished. The current recommended method is the aspiration at a constant flow rate from one naris with gas entrained *via* the other naris or orally (ATS/ERS 2005). An overview of reported normal nNO levels suggests an inverse flow-dependence with higher NO levels being related to lower sampling airflow rates (Antosova et al. 2017; Bartley et al. 1999). In the present study, air was continuously sampled through an olive application tightly inserted inside one nostril at a flow rate of 5 ml/s (i.e., 300 ml/min) as recommended (ATS/ERS 2005). At a flow-rate of 250 ml/min, mean nNO levels between 651 and 1,197 ppb were reported (Ferguson and Eccles 1997) which could be confirmed by our results.

The particular sampling mode is another major influential matter. Nasal aspiration can be performed during breath-holding, tidal-breathing, or oral exhalation against a resistance. Lowest values are reported during quiet exhalation and methods in which there is a turbulence of nasal flow as in tidal-breathing. Highest values are reported in the methods with an elevated velum as during breath-holding or while exhaling against a resistance (de Winter-de Groot and van der Ent 2009). Recently, it has been suggested that calm breathing through the mouth while avoiding any kind of breathing through the nose creates two distinct, independent compartments (oropharynx and nasopharynx) that are not connected to each other. Applying this technique, our results are similar to a mean nNO level of 427 ppb reported by Gelardi et al. (2016) in 32 healthy nonsmoking subjects of the mean age of 30 years. Antosova et al. (2017), using the same approach, have also reported an average nNO value of 379.6 ± 170.4 ppb in the right and 401.6 ± 207.8 in the left nostril (n = 141, mean age 25 years).

Overall, in this study we demonstrate a lower mean nNO level using the technique of tidal-breathing compared to breath-holding. An explanation might be the air with a low NO concentration from the lower respiratory tract diluting the higher concentration in the nasal area. However, during breath-holding the velum keeps elevated, which closes off the nasal passages as demonstrated by a simultaneously assessment of CO_2 (Bartley et al. 1999). In some subjects similarly high nNO levels are detected using both techniques. This finding suggests that the velum may also remain elevated while breathing calmly through the mouth.

Breath-holding improves the reproducibility of nNO measurement. At a flow rate of 500 ml/min and aspiration during breath-holding, the reproducibility of 6.6% at a single point in time, in terms of coefficient of variation, has been demonstrated (Bartley et al. 1999). For three consecutive measurements during breath-holding at a flow rate of 300 ml/min a lower mean intra-subject coefficients of variation of 3.8% has been reported for breath-holding compared to 9.9% using tidal-breathing (de Winter-de Groot and van der Ent 2009). Kharitonov et al. (2005) have demonstrated by applying the breath-holding technique a repeatability of two and three consecutive measurements of 6.2% and 7.7%, respectively, in healthy adults with nNO mean values of about 870 ppb.

Further, we demonstrate that the short- and long-term repeatability are influenced by the method used for nNO measurements. We used the Bland and Altman analysis and evaluated repeatability in terms of coefficient of variation. The variance of the measurements repeated within 4 weeks remained stable indicating the absence of external factors influencing it. Our findings are consistent with the results reported previously. Within-day repeatability of 13.4% and between-day repeatability (1 week later) of 11.8% have been demonstrated at a flow rate of 500 ml/min and aspiration during breath-holding (Bartley et al. 1999). A higher within-day than between-day variability might indicate a systematic diurnal variation. The Bland and Altman analysis also reveals good short- (t = 0–1 h) and long-term (t = 0–24 h) reproducibility for breath-holding at a flow rate of 300 ml/min and is recommended for standardized measurements of nasal NO (de Winter-de Groot and van der Ent 2009). Boot et al. (2007) have reported the reproducibility of nNO levels for 1 day (coefficient of variation 16.5%), up to 7 days (coefficient of

variation 21.5%), and 21 days (38.3%) in patients with allergic rhinitis. In that study, nNO was aspirated during exhalation against a resistance at a flow rate of 300 ml/min. The authors suggest that a decreasing reproducibility over time might be due to subclinical seasonal influences. Repeatability data for day-to-day (coefficient of variation 8.1% morning, 17.5% afternoon), week-to-week (coefficient of variation 12.3%), and seasonal comparisons (21.0%) have been revealed when measuring nNO by application of the flow exhalation technique at a flow rate of 100 ml/min, using a chemiluminescence analyzer (Stark et al. 2007).

So far, there are few data on nNO as an effect marker in follow-up, intervention, or challenge trials. With respect to a possible underlying biorhythm of nNO levels, it is important to consider the time of measurement for repeatable measurements during challenge or longitudinal studies (Dressel et al. 2008; Palm et al. 2000). In order to avoid misinterpretation of time dependent variations, our measurements during the sessions were performed at the same hours. Moreover, control sham conditions were regularly included in our challenge studies. Ambient nNO levels were controlled during exposure with ethyl acrylate. Therefore, any impact of ambient NO on nNO levels as by other authors (Gehring et al. 2009) could be excluded in our study. Overall, the present findings suggest no underlying biorhythm during a 4-h-long exposure.

We observed similar baseline nNO levels in atopic and non-atopic subjects. In the subjects suffering from allergic rhinitis, nNO in the off-allergen-season has been shown not to be significantly different from controls (Henriksen et al. 1999). An association between the nNO level and the presence and type of sensitization is described in another study in which nNO increased with the number of perennial allergens to which subjects were IgE-sensitized (Krantz et al. 2014).

Concerning the usefulness of nNO in terms of a biomarker of inflammation, lower nNO levels are seen when patients with allergic rhinitis were treated with topic steroids compared to non-treated ones (Kharitonov et al. 1997). Also, increasing nNO and fractional nasal (FnNO) are reported under the influence of a specific allergen challenge in subjects with allergic rhinitis and mold problems, respectively (Boot et al. 2007). However, after *Aspergillus fumigatus* and placebo inhalation, FnNO profiles are almost identical (Stark et al. 2005). Taking the effects of a sham challenge into account, we found in the present study a net increase in nNO after constant exposure to 5 ppm ethyl acrylate in atopic subjects. Nasal NO levels have also been shown to fall after a specific challenge (Serrano et al. 2012). A decline in nNO may be caused by acute inflammation-induced nasal congestion, edema, and secretions, leading to a reduction in NO-rich air passage through the paranasal ostia. Recently, we have found that after a constant challenge with 5 ppm of ethyl acrylate, complaints and signs of nose irritation were in a range of weak-to-moderate (Hoffmeyer et al. 2016). Thus, we assume that no severe nasal congestion affecting nNO levels should be considered in this study.

5 Conclusions

Significant increases in nNO could be demonstrated in response to ethyl acrylate challenge with both passive aspiration during breath-holding or calm tidal breathing. The increases were consistent in that nearly all subjects demonstrated a positive net-change and the mean change exceeded the respective methodological coefficients of variation. The findings, restricted to atopic subjects, are in line with our recent study concerning changes in fractional exhaled NO (FeNO) after a constant ethyl acrylate challenge (Hoffmeyer et al. 2017). Our findings suggest that nNO might be a useful effect marker and that the less elaborate tidal-breathing method is sufficient to detect significant changes at a group level. Further research is needed to validate the usefulness of nNO in the evaluation of irritative, non-allergic responses. Given a lower coefficient of variation of breath-holding we assume the advantage of that approach at an individual level.

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

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Airway Obstruction in Sleep Apnea Patients

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Abstract

Obstructive sleep apnea (OSA) is a condition of breathing pathology occurring during sleep, characterized by repeated episodes of upper airway obstruction. The aim of the study was to determine the occurrence of airway obstruction in smoking males with OSA in whom lung function tests had not been performed before. One hundred and four current smokers selected from 1241 patients were enrolled for the research. The subjects included in the study smoked minimum 20 cigarettes a day for at least 10 years. The diagnosis of OSA was confirmed by polysomnography (PSG) in the Sleep Laboratory and subjects were assigned to one of three groups, depending on the severity of OSA. The control group consisted of 30 age-matched male smokers in whom OSA was not confirmed in PSG. Patients from the study and control group scored $\geq$ 11points in the Epworth Sleepiness Scale. Spirometry, impulse oscillometry, and body plethysmography were used to assess pulmonary function. Airflow limitation in subjects of the control group and OSA patients was confirmed. There were no significant differences in the incidence of bronchial obstruction between the control and study groups, and among the patients of various OSA severity. We conclude that the severity of OSA in smokers does not associate with the presence of airway obstruction. However, the increased peripheral respiratory resistance found in oscillometry did relate to a longer smoking time in OSA patients.

Keywords

Airflow limitation · Airway obstruction · Impulse oscillometry · Polysomnography · Pulmonary function · Respiratory resistance · Sleep apnea · Spirometry

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1 Introduction

Obstructive sleep apnea (OSA) is a breathing pathology which occurs during sleep and is characterized by repeated episodes of upper airway obstruction, excessive daytime sleepiness, and is associated with hypoxemia and arousals disturbing the architecture of sleep (Epstein et al. 2009). An objective method of assessing excessive daytime drowsiness is the Epworth Sleepiness Scale (ESS). The questionnaire includes questions about the possibility of falling asleep in common situations of daily living (Buysse et al. 2008). Full-night polysomnography (PSG) is a commonly used test in the diagnosis to determine the severity of OSA and it should be considered in case of patients with significant sleepiness. PSG includes electroencephalography (EEG), electrooculography (EOG), and electromyography (EMG) required for the assessment of sleep stages. Other parameters monitored in a sleep study include the following: electrocardiography (ECG), pulse oximetry, respiratory effort (thoracic and abdominal), sound recordings to measure snoring, and body position (Kushida et al. 2005).

Spirometry is a standard lung function test for diagnosing and staging the airway obstruction. Changes associated with airway obstruction are expressed in the reduction of the ratio of forced expiratory volume in one second to forced vital capacity (FEV_1/FVC), and the ratio of forced expiratory volume in one second to its predicted value (FEV_1pred). A complementary to spirometry in the assessment of airflow limitation in airways is impulse oscillometry (IOS). The respiratory obstruction in this method manifests itself as an increase of total respiratory resistance (R5), proximal respiratory resistance (R20), peripheral respiratory resistance (R5-R20), and more negative value of distal capacitive reactance (X5) (Jaranbäck et al. 2013; Crim et al. 2011). The aim of the study was to determine the incidence of airway obstruction in smoking males with OSA in whom lung function tests had not been performed before.

2 Methods

2.1 Patients

The study protocol was approved by the Ethics Committee of the University of Medical Sciences in Poznan, Poland (approval no. 278/14). All subjects participating in the study patients gave informed consent. One hundred and four current smoking males selected from 1241 patients in whom OSA was confirmed, were enrolled for the research in the Sleep Laboratory of the Department of Pulmonology, Allergology and Respiratory Oncology at the University of Medical Sciences in Poznan, Poland. The mean age of patients was 66.6 ± 9.4 years and their BMI was of 35.5 ± 8.1 kg/m^2. The patients smoked a minimum of 20 cigarettes a day for at least 10 years. The control group consisted of 30 age- and BMI-matched (59.9 ± 10.7 years and 30.2 ± 2.4 kg/m^2, respectively) male smokers (≥ 1 pack-year) in whom OSA had not been confirmed in PSG (Table 1). Excessive daytime drowsiness was assessed with the Epworth

Table 1 Basic characteristics of study subjects

	Control group (*n=30*)	OSA group – all (*n=104*)	Mild OSA (*n=36*)	Moderate OSA (*n=32*)	Severe OSA (*n=36*)
Age (year)	59.9 ± 10.7	66.6 ± 9.4	65.9 ± 9.9	68.1 ± 9.5	71.2 ± 9.2
AHI (episodes/h)	3.2 ± 1.1	36.9 ± 25.0	10.1 ± 2.7	25.4 ± 4.2	56.7 ± 16.5
Body weight (kg)	92.6 ± 12.8	105.9 ± 25.6	87.8 ± 13.6	104.1 ± 40.0	117.3 ± 21.2
Body height (cm)	171.7 ± 10.3	166.5 ± 8.6	174.0 ± 8.9	168.0 ± 11.0	172.7 ± 6.8
BMI (kg/m^2)	30.2 ± 2.4	35.5 ± 8.1	29.0 ± 4.9	36.4 ± 10.8	39.3 ± 6.4

OSA obstructive sleep apnea, *BMI* body mass index

Sleepiness Scale (ESS). This questionnaire includes eight questions about the possibility of falling asleep in common situations of daily living, each scored from 0 to 3. The ESS score represents the sum of individual items, and ranges from 0 to 24. Values $\geq$11 are considered to indicate significant sleepiness and risk of OSA (Faria et al. 2015; Buysse et al. 2008). All the patients received $\geq$ 11 points in the ESS. Based on PSG, the study subjects were assigned to one of three groups, depending on the severity of OSA: mild degree – 36 patients, moderate degree – 32 patients, and severe degree – 36 patients.

2.2 Instrumentation

The diagnosis of OSA was performed by full-night PSG using the Embla S4000-Remlogic polysomnograph with Somnologica Studio 5.0 software (Natus Medical Incorporated – Embla, Pleasanton, CA). Based on the PSG results, stages of OSA were determined as mild apnea-hypopnea index (5$\leq$ AHI < 15) moderate (15 $\leq$ AHI $\leq$ 30) $\geq$, and severe (AHI > 30).

Lung function was assessed with the oscillometric and spirometric methods in both OSA and control groups according to the European Respiratory Society and American Thoracic Society (ERS/ATS) recommendations (Miller et al. 2005). In addition, body plethysmography was performed (MasterScreen Jaeger; Höchberg, Germany). The spirometric measurements included the following ratios: FEV_1/FVC – the forced expiratory volume in one second to the forced vital capacity and FEV_1%pred – the forced expiratory volume in one second to its predicted value, and FVC% pred – the forced vital capacity to its predicted value. The criterion for the diagnosis of bronchial obstruction was a fixed ratio of FEV_1%FVC less than the fifth percentile. Using IOS, the following measurements were assessed: total respiratory resistance at 5 Hz (R5) – comprising extrathoracic, central and peripheral airways, proximal resistance at 20 Hz (R20) – comprising mainly extrathoracic and central airways, and distal capacitive reactance at 5 Hz (X5) – comprising elastic lung and thorax components. The peripheral resistance at 5 Hz minus the one at 20 Hz (R5–R20) was calculated. The criterion of increased respiratory resistance was R5 > 150 % predicted, R20 > 150 %predicted, R5 – R20 >20%, and X5 $\leq$ –0.12, all in kPa s L^{-1}. Restrictive ventilatory defects in all subjects from the control and patient groups were excluded on the basis of total lung capacity (TLC) measurement in body plethysmography, expressed as air volume in the lungs at maximal inflation. The criterion of norm was the value of TLC above the fifth percentile.

2.3 Statistical Elaboration

Continuous data were described as means $\pm$SD, and categorical variables as percentages. The Kruskal-Wallis test followed by Dunn's *post hoc* test, and a Chi-square test were used to compare the subgroups of OSA severity. The relationship between pulmonary function parameters was assessed with Spearman's correlation. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were evaluated. All reported p-values were two-sided. Statistical analyses were performed with STATISTICA 12.0 (Statsoft; Tulsa, OK).

3 Results

The mean age and BMI of OSA patients increased with increasing severity of the disease (Table 1). There was no differences in the prevalence of obstruction (FEV_1%FVC <5th percentile) between the control group and the OSA severity subgroups. The percentage of patients with obstruction determined by the FEV_1%FVC ratio amounted to 43.3% in the control group and 52.3% in the OSA group, and it was similar in the successive severity stages of OSA. However, there was a significant 2-3-fold increase in the percentage of patients with increased values of R5 and R5-R20 with increasing OSA severity compared with the control subjects (Table 2). A higher percentage of patients with increased R5

Table 2 Percentage of subjects in the control group and in each OSA severity subgroup in whom and airway obstruction was detected on the basis of the assessment of the three markers FEV_1/%FVC ratio, R5, and R5-R20

	Controls (*n=30*)	OSA (*n=104*)		
		Mild OSA (*n=36*)	Moderate OSA (*n=32*)	Severe OSA (*n=36*)
FEV_1%FVC ratio	43.3	54.7	48.7	53.1
R5	33.3	66.7	59.4	80.5
R5-R20	32.7	72.2	84.4	86.1

OSA obstructive sleep apnea, *FEV_1* forced expiratory volume in one second, *FVC* forced vital capacity, *R5* resistance at 5 Hz, *R20* resistance at 20 Hz

Table 3 Pulmonary function in control subjects and in patients subdivided into successive OSA severity stages

	Controls (*n=30*)	OSA (*n=104*)			p-value
		Mild OSA (*n=36*)	Moderate OSA (*n=32*)	Severe OSA (*n=36*)	
AHI (episodes/h)	3.2 ± 1.2	10.1 ± 2.7	25.4 ± 4.2	56.7 ± 16.5	0.0220
FVC (L)	4.22 ± 1.26	2.62 ± 1.08	2.10 ± 0.70	4.11 ± 1.33	<0.0001
FEV_1 (L)	3.24 ± 1.13	1.70 ± 1.80	1.10 ± 0.50	2.95 ± 1.34	<0.0001
FEV_1%FVC ratio	79.4 ± 4.8	62.6 ± 12.4	53.4 ± 14.3	55.0 ± 11.3	<0.0001
R5 (kPa s L^{-1})	0.57 ± 0.17	0.62 ± 0.12	1.26 ± 0.22	1.37 ± 0.36	<0.0001
R20 (kPa s L^{-1})	0.30 ± 0.07	0.37 ± 0.09	0.41 ± 0.18	0.34 ± 0.09	<0.0001
R5-R20 (kPa s L^{-1})	0.20 ± 0.09	0.22 ± 0.06	0.70 ± 0.08	0.90 ± 0.23	<0.0001
X5 (kPa s L^{-1})	−0.17 ± 0.08	−0.70 ± 0.50	−0.50 ± 0.40	−0.61 ± 0.35	<0.0001

Data are means ±SD; *OSA* obstructive sleep apnea, *AHI* apnea-hypopnea index, *FEV_1* forced expiratory volume in one second, *FVC* forced vital capacity, *R5* airway resistance at 5 Hz, *R20* airway resistance at 20 Hz, *X5* reactance at 5 Hz. P-values denote significant differences among the subgroups of progressing OSA severity for each variable (Kruskal-Wallis test); *post-hoc* Dunn's pairwise comparisons insignificant

Table 4 Sensitivity and specificity of FEV_1%FVC ratio for the diagnosis of obstructive impairment in obstructive sleep apnea (OSA)

Sensitivity %	87.8
Specificity %	66.7
Positive predictive value %	87.8
Negative predictive value %	66.7

FEV_1 forced expiratory volume in one second, *FVC* forced vital capacity

Table 5 Sensitivity and specificity of R5-R20 for the diagnosis of obstructive impairment in obstructive sleep apnea (OSA)

Sensitivity %	89.4
Specificity %	50.0
Positive predictive value %	80.8
Negative predictive value %	66.7

R5 airway resistance at 5 Hz, *R20* airway resistance at 20 Hz

and R5-R20 could in part be related to a longer smoking time in somehow older subjects in the subgroup of most severe OSA.

Comparison of pulmonary function tests in the control subjects with those in the OSA patients showed a distinct progressive decline of FEV_1% FVC and worsening of the oscillometric variables in successive severity stages of OSA (Table 3). Airway obstruction, increased R5, and particularly R5-R20 indicate that more severe stages of OSA were accompanied by chronic obstructive pulmonary disease (COPD). The sensitivity and specificity of FEV_1%FVC ratio for the diagnosis of obstructive impairment in OSA was 87.8%, and 66.7%, respectively (Table 4) and that of R5-R20 was 89.4% and 50.0%, respectively (Table 5). Comparing the FEV_1%FVC ratio and the R5-R20 assessment shows that the sensitivity of the latter was somehow greater in the detection of airway obstruction. In this study we could not substantiate the presence of any

significant relationships between the AHI and the changes in FEV_1%FVC ratio, R5, R5-R20, or X5.

4 Discussion

The aim of the study was to determine the frequency of ventilatory impairment characterized by airway obstruction in patients with OSA, who actively smoke cigarettes for many years. Current cigarette smoking concerns 3.5 million of Polish adult females (21.0%) and 5.2 million of males (33.5%), a total of 8.7 million adult population (King et al. 2013). We used spirometry and oscillometry as the screening tools to detect the airway obstruction in OSA. A lot of hypotheses were formulated in the literature, which try to associate OSA with obstructive diseases such as asthma or COPD (Hang et al. 2016; Steveling et al. 2014). This study attempts to approach the issue in a different way, i.e., to assess the ventilatory impairment in patients with OSA in the process of a polysomnographic examination. To assess the obstructive impairment, we chose to eliminate patients with restrictive defects. Coexistence of OSA and COPD is called an overlap syndrome (McNicholas 2017). In many cases, the overlap syndrome poses a significant clinical problem, requiring specified diagnostics and treatment (Hang et al. 2016; Budhiraja et al. 2015; Sanders et al. 2003). Coexistence of both diseases ranges from 20% to 30% worldwide, whereas it is presumed to occur only in 1% of the adult population aged over 40 in Poland (Bednarek et al. 2005). COPD detectability in OSA patients ranges from 9% to 29% (De Miguel et al. 2002; Resta et al. 2002; Chaouat et al. 1995). This study demonstrates that the percentage of patients with airway obstruction detected in spirometry by the FEV1%FVC ratio was 52.3% in case of OSA patients, compared with the inappreciably smaller percentage of 43.3% in the control group. In this context, it is worth mentioning a new alternative overlap syndrome consisting of the coexistence of OSA and asthma. This issue, which was beyond the scope of the present study, has been innovatively tackled in a recent paper by Khatri and Ioachimescu (2016).

A well-documented risk factor for OSA progression is obesity (Chokroverty et al. 2005). In this study we confirmed the presence of obesity in both OSA and control groups. The greatest BMI was found in patients with moderate-to-severe OSA, although no statistically significant differences between the severity of OSA and BMI were demonstrated (Table 1). Obesity often accompanies also COPD. The Polish epidemiological studies have demonstrated the presence of COPD, on average, in 7.5% of the Polish population aged 60–70 (Pływaczewski et al. 2008). The OSA prevalence is akin to that reported in the population studies in which age was used as a significant factor of risk of OSA occurrence (Mariani et al. 2013; Clarenbach et al. 2011; Ogawa et al. 2004).

In this study, aside from classical spirometry, we used oscillometry for the determination of ventilatory impairment. Oscillometric variables that assess the total respiratory resistance (R5) and peripheral airways resistance (R5-R20) seem to offer a better sensitivity than spirometry for detection of airway obstruction (Piorunek et al. 2015; Oosteveen et al. 2003; Schulz et al. 2013; Winkler et al. 2009). We demonstrate that the severity of OSA in smokers does not associate with the presence of airway obstruction. However, the percentage of patients with increased peripheral respiratory resistance (R5-R20) was related to a longer smoking time stemming from the older patients' age. The same phenomenon has been reported by other researchers, who, in contrast to the present study, looked for OSA in patients already having COPD, and not for obstructive impairment in patients already having OSA (Guerreiro et al. 2015; Anderson and Lipworth 2012; Allen and Baxter 2009; Cooper 2005).

In conclusion, oscillometry, alongside spirometry, should be additionally recommended in the diagnostics of sleep disordered breathing. Such approach enables a precise assessment of the presence of obstructive ventilatory impairment, particularly in subjects at risk of such impairment, for instance, in cigarette smokers. Pulmonary function assessment may help commence the appropriate and early treatment.

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

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Long-Term Exposure to Ambient Air Pollution in Childhood-Adolescence and Lung Function in Adulthood

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Abstract

The aim of the study was to evaluate the effect of air pollution in the dwelling place during childhood-adolescence on respiratory function in early adulthood. The study was conducted in 220 female and 160 male university undergraduates in the cities of Cracow and Wroclaw in Poland and consisted of spirometry to assess lung function. The subjects' exposure to pollution during childhood-adolescence was assessed from the data acquired by the Polish Chief Inspectorate for Environmental Protection. We found differences in all spirometry variables depending on benz[a]piren exposure, in FVC% and FEV_1/%FVC depending on $PM_{2.5}$ content, and in FVC% depending on NO_2 content. Statistically significant differences in spirometry variables were also found in relation to the degree of urbanization of the place of living during the early life period in question. The higher the urbanization, the higher is FEV_1% and FCV%, and the lower FEV_1/%FVC. Additionally, undergraduates of Cracow University had worse lung function compared to those of Wroclaw University. In conclusion, air pollution in the dwelling place during childhood-adolescence has an impact on lung function in early adulthood, independently of the current exposure to pollutants.

Keywords

Adolescence · Adulthood · Air pollution · Lung function · Particular matters · Spirometry · Urbanization

1 Introduction

Chronic respiratory diseases are among the most common health problems. Such diseases reduce lung capacity and respiratory ability, impairing functions of other systems and leading to comorbid conditions. According to WHO (2014), hundreds of millions of people suffer every day from respiratory diseases. An important part of the diagnostics is spirometry tests that enable the early detection of a lung function decrement. Forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) are the two essential variables in the objective assessment of respiratory health. These variables are considered the

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early indicators of respiratory inflammation and also relate with cardiovascular diseases.

Air pollution is one of the most important environmental determinants of pulmonary function. According to a report by the European Environment Agency (2015), the most harmful substances detected in the air include suspended particulate matter, ozone, nitrogen dioxide, and benzo[a]pyrene; the last being a chemical compound present mainly in Eastern Europe. Poland is listed among countries where most pollutants exceed acceptable limits.

Acute respiratory health effects of worsening air quality are well established (Nkosi et al. 2016; Anderson et al. 2012; Sunyer 2009; Dominici et al. 2006). Numerous studies have evaluated the effects of long-term exposure to air pollution, which affects mostly children and adolescents as these populations are particularly sensitive to environmental factors (Perera et al. 2012; Wang et al. 2009; Calderón-Garcidueñas et al. 2008; Dales et al. 2008; Suglia et al. 2008). Children are more likely than adults to spend time outdoors, where the concentration of pollutants is greater, and their respiratory system is not yet fully developed. A greater ventilation rate and mouth-breathing may pull air pollutants deeper into the children's lungs, thereby making clearance slower and more difficult (Bateson and Schwartz 2007). Their immune system also is immature, which promotes respiratory infections.

Both longitudinal and cross-sectional studies show that long-term exposure to air pollution in childhood is associated with a retardation of the respiratory system development (Gauderman et al. 2002, 2004; Horak et al. 2002). However, some authors have failed to confirm such a relationship (Hoek et al. 2012; Nicolai et al. 2003). A Greek study has demonstrated that particulate air pollution has a significant impact on the development of nasal, but not lung, respiratory function (Spyratosa et al. 2015). Nonetheless, most studies point to a relation between long-term exposure to air pollution and respiratory health. Specifically, changes in spirometry variables and the intensity of respiratory symptoms are reported in the context of the atmospheric air quality. Pollutant emissions cause a deterioration of respiratory function in children and adolescents (Rice et al. 2016; Schultz et al. 2016), while a reduction in the emissions improves the function (Gauderman et al. 2015). A detrimental effect of pollutants on respiratory health may be present even when their content is below the current permissible limits (Moshammer et al. 2006).

Notwithstanding the respiratory detriment of air pollution in childhood above outlined, the adulthood consequences of the pollution are not fully documented; permanent lung damage or retardation in lung development is a possibility. Therefore, the present study seeks to examine the respiratory function in early adulthood in relation to air pollution in the current dwelling place and the dwelling place during childhood-adolescence of the same subjects.

2 Methods

2.1 Study Subjects

The study protocol was approved by a local Ethics Committee of the Jagiellonian University in Cracow, Poland. Data were collected following the ethical principles as stated in the Declaration of Helsinki for Human Research. The measurements were taken in March 2016 and January 2017. The study included 220 women and 160 men of the mean age of 20.5 ± 1.2 (SD) years, with the median of 20 years of age. There were 250 undergraduates of the Jagiellonian University in Cracow and 130 undergraduates of the University of Environmental and Life Sciences in Wroclaw, Poland. All participating subjects were free of chronic diseases other than possible food allergy and allergic rhinitis. All lived in the respective cities for at least one year.

2.2 Pulmonary Function, Dwelling Place, and Air Pollution

FEV_1 and FVC were measured, taking into consideration the best of three forced maneuvers. Data were expressed in the percentage of predicted values (%pred). In addition, the ratio of FEV_1/% FVC was calculated. Lung function was tested using a portable MIR Spirolab III device (Medical International Research; Rome, Italy).

The information on the number of years spent in the current dwelling place and the dwelling place during childhood-adolescence was collected by means of a questionnaire. The subject's dwelling place before entering a university was specified in categories defined according to the air pollution level (1. area of low air pollution; 2. area of average air pollution; and 3. area of high air pollution) and the degree of urbanization (1. city – over 100,000 inhabitants; 2. town – less than 100,000 inhabitants; and 3. village).

The subject's dwelling place during the childhood years also was stratified into three categories, taking into account the air quality to which the area in question was classified during a period of no less than 10 years. Class 1 refers to the zones below the lower cut-off limit; Class 2 – zones between the lower and upper cut-off limits; and Class 3 – zones above the upper cut-off limit. The annual lower and upper cut-off limits were as follows: for NO_2 30 and 40 μg/m^3, for benz[a]piren 4 and 5 μg/m^3, and for CO, for which no area qualified to Class 3, these limits were 0.5 and 1.0 mg/m^3, respectively. Concerning the $PM_{2.5}$ and PM_{10} particulate matter, all zones qualified to Class 3, i.e., the annual mean values exceeded 15 and 30 μg/m^3, respectively. The PM-related stratification considered three categories: moderate (<20 μg/m^3 for $PM_{2.5}$ and <40 μg/m^3 for PM_{10}), high (≥21 <25 μg/m^3 for $PM_{2.5}$ and ≥41 <50 μg/m^3 for PM_{10}), and very high level (>25 μg/m^3 for $PM_{2.5}$ and >50 μg/m^3 for PM_{10}). It was based on the measurements made over the last 16 years and expressed medians and tertiles.

A division into areas with different pollution levels was made on the basis of data acquired by the Chief Inspectorate for Environmental Protection in Poland between the years 2000 and 2016 (http//:www.gios.gov.pl).

2.3 Statistical Elaboration

Data were presented as means ± SD. Distribution of quantitative data was checked using the Shapiro-Wilk test. A *t*-test was used to evaluate differences in spirometry variables in relation to gender and the current dwelling place (Cracow or Wroclaw). Multivariate analysis of variance MANOVA was used to evaluate differences in lung function depending on air quality and the degree of urbanization of the dwelling place during childhood-adolescence and to verify the interactions between these variables. A p-value of <0.05 defined the statistically significant differences.

3 Results

Spirometry results, separately for women and men, are presented in Table 1. The results were slightly, albeit insignificantly, greater in men, both in the absolute values and in reference to standards. Further evaluation was thus performed for the whole group, regardless of gender.

Current residents of Cracow had a significantly lower FEV_1% and FVC%, while displaying a higher FEV_1/%FVC ratio than those living in Wroclaw (Table 2). Of note, both cities are characterized by a high level of air pollution and classified as air quality Class 3 in 2015–2016, taking into consideration $PM_{2.5}$, $PM_{10,}$ benz[a]piren and NO_2. In case of CO, Wroclaw was classified as Class 1 and Cracow as Class 2. In case of benz[a]piren Wroclaw was classified as Class 2, while Cracow as Class 3. Overall, greater average annual content of the above-mentioned pollutants was observed in Cracow than in Wroclaw. This study was, in part, conducted in January of 2017 when Cracow was shrouded in smog for several days, which, in all likelihood, influenced the results, as the spirometry variables were appreciably worse compared with the same period a year before.

The degree of urbanization of the dwelling place during childhood-adolescence was significantly associated with spirometry variables. A greater FEV_1 and FCV, and a lower FEV_1/FVC were noted among the undergraduates from rural areas than among those from urban areas (Table 3). In addition, the degree of urbanization significantly altered the relationship between the level of air pollutants and respiratory function. Variations in respiratory function across areas with different pollution were greater in large cities than in villages (Table 4).

Table 1 Lung function of surveyed undergraduates

Variable	Total	Females	Males	p
FEV_1 (L)	3.17 ± 0.46	3.14 ± 0.44	3.20 ± 0.47	0.307
FEV_1 (%pred)	97.3 ± 13.2	95.2 ± 12.2	99.0 ± 14.1	0.519
FVC (L)	3.64 ± 0.53	3.58 ± 0.53	3.66 ± 0.53	0.409
FVC (%pred)	97.1 ± 13.0	95.4 ± 12.1	98.1 ± 14.1	0.813
FEV_1/%FVC	89.2 ± 10.3	87.1 ± 9.2	90.2 ± 9.3	0.813

Data are means ±SD; p-values concern inter-gender differences based on a *t*-test

Table 2 Spirometry variables depending on air quality in the dwelling place during childhood-adolescence

Factor	Category	n	FEV_1 (%pred)	FVC (%pred)	FEV_1/%FVC
Current dwelling place	Wroclaw	130	98.3 ± 13.1	99.1 ± 13.2	84.1 ± 8.0
	Cracow	250	91.4 ± 11.1	92.4 ± 14.0	88.7 ± 9.1
			p = 0.043	p = 0.042	p = 0.049
CO	Class 1	178	101.5 ± 10.7	104.3 ± 11.6	85.9 ± 7.4
	Class 2	202	94.2 ± 16.2	95.1 ± 14.2	89.0 ± 9.1
			p = 0.189	p = 0.110	p = 0.363
Benz[a]piren	Class 1	110	99.3 ± 16.3	103.2 ± 14.2	84.8 ± 6.9
	Class 2	156	95.4 ± 14.5	95.3 ± 13.4	87.6 ± 7.1
	Class 3	114	87.4 ± 0.18	88.4 ± 15.2	91.9 ± 11.0
			p = 0.049	p = 0.009	P = 0.043
NO_2	Class 1	165	99.1 ± 16.1	100.2 ± 14.1	84.9 ± 7.6
	Class 2	124	94.9 ± 14.3	94.4 ± 13.4	88.5 ± 8.2
	Class 3	91	92.1 ± 18.1	90.1 ± 15.0	90.2 ± 8.6
			p = 0.619	P = 0.072	p = 0.664
$PM_{2.5}$	1. moderate level	125	98.4 ± 12.3	102.1 ± 13.2	84.7 ± 7.3
	2. high level	128	95.6 ± 12.2	95.2 ± 12.2	88.5 ± 8.7
	3. very high level	127	93.5 ± 17.8	89.2 ± 16.1	91.5 ± 12.0
			p = 0.619	p = 0.010	p = 0.046
PM_{10}	1. moderate level	123	102.2 ± 12.3	103.1 ± 17.9	85.0 ± 8.5
	2. high level	130	95.4 ± 19.1	97.3 ± 16.4	87.1 ± 9.0
	3. very high level	127	93.3 ± 19.7	94.2 ± 18.0	88.9 ± 9.2
			p = 0.189	p = 0.110	p = 0.363

Data are means ±SD; p-values based on MANOVA

Table 3 Spirometry variables depending on the degree of urbanization of the dwelling place during childhood-adolescence

	n	FEV_1 (%pred)	FVC (% pred)	FEV_1/%FVC
Cities with ≥100,000 inhabitants	162	85.2 ± 18.9	87.3 ± 13.2	93.6 ± 9.0
Other cities and towns	75	96.4 ± 16.1	92.3 ± 15.2	87.3 ± 8.1
Villages	143	100.2 ± 17.2	104.2 ± 16.1	86.2 ± 7.1
		p = 0.048	p = 0.010	p = 0.036

Data are means ± SD; p-values based on MANOVA

Table 4 Differences in lung function depending on both degree of urbanization and air quality in the dwelling place during childhood-adolescence

Factor 1	Factor 2	FVC (% pred)		FEV_1 (% pred)		FEV1/% FVC	
		F	p	F	p	F	p
Urbanization of the dwelling place during childhood-adolescence	Current dwelling place	3.53	0.041	3.96	0.041	2.89	0.045
	CO	4.46	0.022	2.53	0.044	3.01	0.043
	Benzene	4.13	0.025	4.01	0.026	4.13	0.038
	NO_2	3.24	0.031	4.33	0.038	3.96	0.345
	$PM_{2.5}$	4.56	0.019	3.99	0.040	4.22	0.021
	PM_{10}	2.92	0.364	2.76	0.046	3.99	0.040

p-values based on MANOVA

4 Discussion

The findings of this study were that the presence of benz[a]piren in the dwelling place during childhood-adolescence had a significant impact on all spirometry variables investigated in the same subjects in adulthood. The $PM_{2.5}$ content influenced FVC, FEV_1/%FVC, and the NO_2 content influenced FVC. Adverse effects of air pollution on lung development in children are well-documented. Particulate matter, sulphur dioxide, carbon monoxide, carbon dioxide, and benz[a]piren pose the highest risk for respiratory health among substances suspended in the air. In the present study we set out to assess the effects on respiratory function of exposure to such substances during childhood-adolescence, delayed in time to early adulthood. Although not all the substances tested had a significant impact on spirometry variables, there was a clear tendency for FVC% and FEV_1% to decrease, and FEV_1/%FVC to increase in adulthood, with a greater content of pollutants in the dwelling place present during childhood-adolescence. The greatest changes in spirometry variables were noted in relation to the ambient concentration of benz[a]piren. We also observed that $PM_{2.5}$ had a greater effect on spirometry variables than that of PM_{10}. These findings are in line with other reports pointing to a more detrimental effect on respiratory health of the finer PM (Zwozdziak et al. 2016).

Consequences of early life exposure to air pollution include diminished lung function and increased susceptibility to acute respiratory illness and asthma (Bateson and Schwartz 2007). The findings of several large cohort studies demonstrate a relationship between air quality and respiratory development. The Harvard 24 cities study, which covered more than 13,000 children aged 8–12, has demonstrated that the prevalence of abnormal lung function rises with increased pollution, especially with $PM_{2.5}$ level (Dockery et al. 1996; Raizenne et al. 1996). Likewise, longitudinal studies conducted over a 4-year period among children living in California have demonstrated a negative correlation between FVC and FEV1, and airborne $PM_{2.5}$ (Gauderman et al. 2002; Gauderman et al. 2000). Living in an environment of poor air quality is associated with lung development retardation. More recent research has confirmed that a long-term improvement in air quality has a positive effect on lung-function growth during adolescence, especially at ages 11 to 15 years (Gauderman et al. 2015). However, it is unknown whether exposure to high levels of pollutants during childhood leads to long-lasting effects, which would also be apparent in adulthood. The literature data fail to ascertain whether the differences in spirometry variables in adulthood are a consequence of a lower level of lung development in childhood-adolescence or an effect of the current exposure to air pollution (Ackermann-Liebrich et al. 1997).

Lung function gradually develops throughout the childhood-adolescence. In girls, respiratory development is completed around the age of 18; in boys about 2 years later (Burrows et al. 1983;

Wang et al. 1993). It is believed that exposure to adverse environmental factors during childhood-adolescence results in abnormal development. The findings of the Swedish birth cohort BAMSE study in 2278 children demonstrate that exposure to car traffic pollution in infancy may have a remote detrimental effect on respiratory function at 16 years of age (Schultz et al. 2016). Another study demonstrates that a reduction in car exhaust gas emissions, cannot much improve, if at all, diminished spirometry variables in otherwise healthy adults (Boogaard et al. 2013), although the issue is contentious as some other studies show that a reduction in PM_{10} may attenuate the decline in lung function related to airborne exposure to PM_{10} (Downs et al. 2007). Studies in animal models have largely confirmed that lung damage during a sensitive developmental period remodels the respiratory tract structure and, as a result, increases susceptibility to respiratory diseases in the future (Fanucchi et al. 2000, 2004).

On the other side, some studies are inclined to conclude that the impairment of lung function in adults results from the current exposure to pollutants. That is confirmed, *inter alia*, by a recent Swiss research that has shown that living in highly polluted areas leads to impairment of respiratory function. The data from the European Study of Cohorts for Air Pollution Effects (ESCAPE) covering more than 7,500,000 people has demonstrated a slight negative correlation between air pollution and long-term pulmonary function. In that study, poor air quality failed to cause a long-lasting downturn in spirometry variables, but increased NO_2 and/or PM_{10} levels did associate with slightly lower both FVC and FEV1 (Adam et al. 2015). It has been observed that spirometry variables can change relatively quickly as air quality varies in either direction in both children and adults, which particularly concerns airborne particulate matter (Boogaard et al. 2013; Cesaroni et al. 2012; Downs et al. 2007; Schikowski et al. 2005; Avol et al. 2001; Ackermann-Liebrich et al. 1997). That was confirmed in the present study as we found that individuals from Cracow tested during the smoggy period had evidently worse spirometry variables than those who had been tested one year earlier in the smog-free environment.

A limitation of this study was a broad definition of air pollution level during the period of subjects' growth and development, i.e., in childhood-adolescence. On the other side, the advantage of the study was a largely homogenous group of subjects who were age-matched and free of overt diseases. The study also spanned an extended period of 10 years. In addition, the participants were university undergraduates and were not exposed to substances posing respiratory hazard, other than ambient air pollution. Significant differences in air quality between city districts were controlled and taken into account in data evaluation. Thus, we believe the study has demonstrated that exposure to air pollution in the early stages of development has a long-term impact on lung function noticeable in adulthood.

In conclusion, this study suggests that the level of lung function in adulthood may have to do with both air pollution in the dwelling place during childhood-adolescence and the current exposure to pollutants. Poor air quality during the developmental age presumably retards lung growth, which is reflected in lower values of lung function variables in adulthood. Exposure to air pollution during childhood-adolescence has an impact on lung function in adulthood, independently of the current exposure. Lung function deterioration seems further augmented in adulthood due to the current pollution-related impact. Thus, pollution affects respiratory health irrespective of lung age, with the possible potentiating overlap of the early and late damage.

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Molecules of Damage-Associated Patterns in Bronchoalveolar Lavage Fluid and Serum in Chronic Obstructive Pulmonary Disease

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Abstract

Chronic exposure to detrimental environmental factors may induce immunogenic cell death of structural airway cells in chronic obstructive pulmonary disease (COPD). Damage-associated molecular patterns (DAMPs) is a family of heterogeneous molecules released from injured or dead cells, which activate innate and adaptive immune responses on binding to the pattern recognition receptors on cells. This study seeks to define the content of DAMPs in the bronchoalveolar lavage fluid (BALF) and serum of COPD patients, and the possible association of these molecules with clinical disease features. Thirty COPD in advanced disease stages were enrolled into the study. Pulmonary function tests, arterial blood gas content, 6-minute walk test, and BODE index were assessed. The content of DAMPs was estimated using the commercial sandwich-ELISA kits. We found differential alterations in the content of various DAMP molecules. In the main, BALF DAMPs positively associated with age, forced expiratory volume in one second (FEV1), and residual volume (RV); and inversely with PaO_2, residual volume/total lung capacity (RV/TLC) ratio, and the disease severity staging. In serum, DAMPS positively associated with the intensity of smoking and inversely with age, PaO_2, and TLC. In conclusion, DAMPs are present in both BALF and serum of COPD patients, which points to enhanced both local in the lung environment as well as systemic pro-inflammatory vein in this disease. These molecules appear involved with the lung damage and clinical variables featuring COPD. However, since the involvement of various DAMPs in COPD is variable, the exact role they play is by far unsettled and is open to further exploration.

Keywords

Bronchoalveolar lavage fluid · COPD · Damage-associated molecular patterns · Immune response · Lung damage · Proinflammatory state · Pulmonary function · Serum · Smoking

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1 Introduction

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death in the world. It is anticipated that the COPD occurrence will increase in the next decades because of continued exposure to risk factors and the aging population. The inflammatory process and exposure to various environmental factors remain pivotal in the pathogenesis of COPD (Vaguliene et al. 2013). Chronic airway inflammation in COPD is characterized by activation of the innate immune system, as defined by increased numbers of neutrophils, macrophages, natural killer cells, and mature dendritic cells in the lung tissue and the airway lumen. Also, the adaptive immune system is activated in COPD, as defined by lung infiltration of CD8 T cells, B cells, and both Th17 and Th1 types of CD4 T cells, along with a decrease in regulatory T cells in the airways (Pouwels et al. 2014). These cells release pro-inflammatory mediators and enzymes and interact with structural cells in the airways, lung parenchyma, and pulmonary vasculature (GOLD 2010).

Danger signals or damage-associated molecular patterns (DAMPs), also known as alarmins, are molecules released from injured or dead cells. DAMPs are conducive to the inflammatory response and act to alarm the immune system by activation of pattern recognition receptors (Matzinger 1994). DAMPs, released in response to cigarette smoke-induced airway epithelial cell injury, trigger the innate and adaptive immune reactions that are part of the COPD pathogenesis (Pouwels et al. 2016). Inhalation of toxic gases and particles causes damage and subsequent cell death of airway epithelial cells (Messner et al. 2012). The main pathway of immunogenic cell death, induced by chronic cigarette smoke exposure, remains still unknown. Upon cell death, DAMPs, e.g., high-mobility group box-1 (HMGB-1), heat shock proteins (HSPs), and S100 protein, are released, which activates pattern recognition receptors, such as toll-like receptors on adjacent epithelial cells, and innate and adaptive immune cells (Heijink et al. 2015; Pouwels et al. 2014). As a result, pro-inflammatory cytokines are released, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-8, and type I interferon (IFN) (Hirsiger et al. 2012; Krysko et al. 2012; Vande Berghe et al. 2006). These cytokines, in turn, activate and attract cells of the innate immune system either directly or indirectly through a release of chemokines or upregulating adhesion receptors.

DAMPs are divided into several subclasses depending on their main subcellular localization (Pouwels et al. 2014). These subclasses consist of DAMPs derived from the cytoplasm (e.g., HSP, S100 protein, or galectin), subcellular organelles (e.g., the nucleus (HMGB1), endoplasmic reticulum (calreticulin), mitochondria, or the extracellular matrix (versican, fibronectin, and hyaluronan). Although the role of DAMPs in the COPD pathogenesis has been extensively studied in experimental models, the reports on the relation of these molecules to the course of COPD in humans are scarce (Sobh et al. 2017; Ünver et al. 2016; Ankersmit et al. 2012; Hacker et al. 2009). Therefore, this study seeks to investigate the presence of DAMPs in the broncholaveolar lavage fluid (BALF) and serum of COPD patients and the possible association of these molecules with clinical signs of the disease.

2 Methods

2.1 Study Population

We obtained the approval to conduct this study from the Ethics Committee of Poznan University of Medical Sciences in Poland. Patients signed written informed consent to participate in the study. The study group consisted of 30 COPD patients (24 males, 6 females) of the mean age of 66.6 ± 1.5(SE) years (range of 52–83 years), and body mass index (BMI) of 26.6 ± 1.0 kg/m^2, range 15.6–39.0 kg/m^2). The patients were either former or current smokers (37.7 ± 4.1 pack/years; range 4–98 pack/years, $n = 27$). The diagnosis of COPD was made according to GOLD criteria (GOLD 2010). The exclusion criteria were the following: other pulmonary disorders, like asthma, tuberculosis, pulmonary thromboembolism or interstitial

pulmonary lesions, and contraindications to perform fiberoptic bronchoscopy or pulmonary function tests.

2.2 Pulmonary Function Tests

The patients performed spirometry, body plethysmography, and the diffusion lung capacity of carbon monoxide (DLCO). Tests were conducted a Master Screen Body/Diffusion Jaeger device (Erich Jeager GmbH, Wurzburg, Germany). Spirometry was performed 15–30 min after inhalation of 400 μg of salbutamol. The results of pulmonary function tests were shown as the percentage of predicted values (% pred). Post-bronchodilator forced expiatory volume in 1 s (FEV1)/forced vital capacity (FVC) ratio <0.70 defined the airflow limitation. We employed the GOLD (2010) division of the severity of airflow limitation into four grades: 1 – mild, 2 – moderate, 3 – severe, and 4 – very severe. The BODE index (B – BMI, O – obstruction, D – dyspnea, and E – exercise) was calculated (Cote and Celli 2005) and dyspnea severity was assessed with the Modified British Medical Research Council (mMRC) scale. The distance achieved in a 6-minute walk test (6MWT) was measured to assess exercise capacity (Pinto-Plata et al. 2004).

Arterial blood samples of 1 ml were drawn from a radial artery after 5-min rest in the sitting position to investigate the blood gas content. Venous blood samples of 9 ml were drawn from an antecubital vein into tubes without an anticoagulant, were centrifuged at 2,500 rpm for 10 min at 4 °C, and the serum obtained was frozen at −70 °C until use.

2.3 Fiberoptic Bronchoscopy and BALF

All patients underwent routine fiberoptic bronchoscopy for diagnostic purposes. BAL fluid samples were collected according to international guidelines (Meyer et al. 2012; Technical recommendations and guidelines for bronchoalveolar lavage (BAL) 1989). Topical lignocaine and intravenous fentanyl and propofol anesthesia were used. The bronchoscope was wedged in the segmental or subsegmental bronchus of the middle lobe of the right lung. The bronchus was lavaged with 50 ml aliquots of sterile saline solution of 37 °C and the fluid was aspirated. Two further 50 ml aliquots of saline were instilled and aspirated in the same way (Chciałowski et al. 2011; Kelsen et al. 1992). The initial preparation of BALF samples consisted of sterile filtration to remove mucus, blood clots, and tissue fragments, followed by the concentration of cells through centrifugation at 1,800 rpm for 10 min. Supernatant was removed, frozen and allocated for the evaluation of DAMPs concentration.

The ethical permission received to conduct this study did not allow for performing bronchoscopy in healthy subjects, which excluded the possibility of having a control group and constituted a limitation of the study.

2.4 DAMPs Assessment

Samples of serum and of BALF supernatants, frozen at −80 °C, were thawed directly before the parallel measurement of DAMPs, using sandwich ELISA tests (Wuhan USCN Business Co, China). The following molecules were assessed: defensine beta-2 (DEF-2) (pg/ml), high-mobility group box-1 (HMGB-1) (pg/ml), heat shock protein-27 (HSP-27) (ng/ml), galectin-1 (GAL-1) (pg/ml), galectin-3 (GAL-3) (pg/ml), and galectin-9 (GAL-9) (pg/ml). The surfactant protein A (SP-A) and hyaluronic acid (HA) were measured with ELISA Kits for Human Pulmonary Surfactant-Associated SP-A (BioVendor Research and Diagnostic Products; Brno, Czech Republic) and for HA (TECOmedical AG; Sissach, Switzerland) (ng/ml). All tests were performed according to the manufacturers' instructions. Serum samples were diluted with a buffer as follows: 1:10 for DEF-2 and for SP-A, 1:50 for HA, and 1:500 for HMGB-1. In addition, BALF samples were diluted 1:100 for the evaluation of SP-A. Accordingly diluted standard samples served for the creation of calibration curves. After stopping the reaction with H_2SO_4

absorbance at 450 nm was measured with a microtitration ELISA reader (Multiskan Bichromatic System; Labsystems Inc., Helsinki, Finland) to determine the concentration of molecules.

2.4.1 Statistical Evaluation

Data are shown as means ±SE. Normality of data distribution was assessed with the Shapiro-Wilk test. A p-value <0.05 defined statistically significant differences. Pearson's correlation coefficient was used to assess the relationship between DAMPs in BALF and in serum and the clinical variables. The evaluation was performed using a commercial Statistica v10.0 package (StatSoft, Tulsa, OK).

3 Results

Patients' functional characteristics are displayed in Table 1, and the concentration of DAMPs in the BALF and serum in Tables 2 and 3, respectively.

In BALF, there were significant inverse associations between HSP-27 and RV/TLC ($r = -0.41$; $p = 0.026$), HSP-27 and GOLD stages I-IV ($r = -0.48$; $p = 0.007$), HSP-27 and BODE index ($r = -0.38$; $p = 0.045$), and GAL-1 and PaO_2 ($r = -0.38$; $p = 0.018$). On the other hand, HSP-27 positively associated with FEV1 ($r = 0.45$; $p = 0.012$), GAL-1 with both RV ($r = 0.39$; $p = 0.040$) and BODE index ($r = 0.39$; $p = 0.040$), and SP-A with age ($r = 0.37$, $p = 0.042$).

In serum, there were significant inverse associations between HSP-27 and age ($r = -0.39$; $p = 0.035$), GAL-1 and both PaO_2 ($r = -0.41$; $p = 0.029$) and SaO_2 ($r = -0.46$; $p = 0.011$), HMGB-1 and SaO_2 ($r = -0.41$; $p = 0.024$), and SP-A and TLC ($r = -0.38$; $p = 0.041$). In addition, SP-A positively associated with the number of pack/years in smokers ($r = 0.40$; $p = 0.038$).

Table 1 Patients' functional characteristics

	n	Mean ± SE	Min–Max
FEV1 (% pred)	30	49.8 ± 3.8	22.1–93.2
FVC (% pred)	30	70.9 ± 3.6	39.8–86.8
RV (% pred)	29	205.7 ± 14.7	28.4–423.8
TLC (% pred)	29	120.5 ± 4.8	67.2–188.5
RV/TLC (%)	29	63.1 ± 2.8	16.1–87.4
DLCO (%)	26	44.7 ± 4.4	15.5–94.5
SaO_2 (%)	30	94.0 ± 0.4	90.4–96.6
PaO_2 (mmHg)	30	67.8 ± 1.1	57.7–80.5
6MWT (m)	30	326.3 ± 24.1	60–545
Bode (0–10)	30	3.8 ± 0.5	0–9
CRP (mg/l)	27	4.7 ± 0.8	0.9–18.6

FEV1 forced expiratory volume in one second, *FVC* forced vital capacity, *RV* residual volume, *TLC* total lung capacity, *DLCO* diffusion capacity of carbon dioxide, *SaO_2* arterial oxygen saturation, *PaO_2* arterial oxygen pressure, *6MWT* 6-minute walking test, *CRP* C-reactive protein

4 Discussion

Different forms of cell death, e.g., apoptosis, necrosis, and necroptosis, may cause distinct signatures of alarmins or damage-associated molecules (DAMPs) released into the extracellular matrix. Regulated forms of cell death encompass both apoptosis, a form of programed and caspase-dependent cell death, and necroptosis. Non-regulated cell death encompasses accidental necrosis, when subjected to physical-chemical injuries disrupt through uncontrolled physical events, triggering cellular components into the microenvironment. Necrotic and necroptotic cell death are the main, but not the only forms of cell death leading to DAMPs release (Pouwels et al.

Table 2 DAMPs content in bronchoalveolar fluid (BALF)

	n	Mean ± SE	Min–Max
DEF (pg/ml)	30	39.2 ± 3.8	5.7–83
HMGb-1 (pg/ml)	30	616.3 ± 40.1	353–1,002
HSP-27 (ng/ml)	30	1.4 ± 0.5	0–13.6
SP-A (ng/ml)	30	1.6 ± 0.6	0–14.1
HA (ng/ml)	30	908.7 ± 153.5	36–3,081
GAL-1 (pg/ml)	29	50.1 ± 5.5	1–74
GAL-3 (pg/ml)	29	0.3 ± 0.1	0–2.2
GAL-9 (pg/ml)	29	0.9 ± 0.3	0–5.6

DEF defensine-beta, *HMGb-1* high-mobility group box-one, *HSP-27* heat shock protein-27, *SP-A* surfactant protein A, *HA* hyaluronic acid, *GAL-1* galectin-1, *GAL-3* galectin-3, *GAL-9* galectin-9

Table 3 DAMPs content in serum

	n	Mean ± SE	Min–Max
DEF (pg/ml)	30	589.2 ± 109.0	247–3,094
HMGb-1 (pg/ml)	30	643.4 ± 24.2	400–1,027
HSP-27 (ng/ml)	30	0.4 ± 0.1	0–2.6
SP-A (ng/ml)	30	5.0 ± 0.3	2.4–8.6
HA (ng/ml)	30	132.8 ± 23.8	0–573
GAL-1 (pg/ml)	29	1171.8 ± 1167.4	0.192–33,859
GAL-3 (pg/ml)	29	4.6 ± 0.8	0.2–18.7
GAL-9 (pg/ml)	29	7.9 ± 1.6	0–29.5

DEF defensine-beta, *HMGb-1* high-mobility group box-one, *HSP-27* heat shock protein-27, *SP-A* surfactant protein A, *HA* hyaluronic acid, *GAL-1* galectin-1, *GAL-3* galectin-3, *GAL-9* galectin-9

2014; Kaczmarek et al. 2013). During early apoptosis, most DAMPs are retained in apoptotic bodies and phagocytized before they bind to pattern recognition receptors. Yet during secondary necrosis, DAMPs can be released (Kono and Rock 2008). Secondary necrosis occurs when apoptotic cells are not cleared sufficiently by phagocytosis. Such process has been observed in COPD (Krysko et al. 2010). Hypoxia, an accompaniment of COPD also causes immunogenic cell death and subsequent DAMPs release (Gallo and Gallucci 2013).

In this study we investigated DAMPs that are released from injured or dead cells. We assessed the level of these molecules in the BALF, as a reflection of a local inflammatory microenvironment, and in the serum, as a sign of systemic inflammation. In addition, we assessed the association between these molecules and clinical functional variables in COPD. The findings are in line with those of other previous studies demonstrating that DAMPs content increases in both fluid compartments investigated in the course of COPD (Sobh et al. 2017; Ünver et al. 2016; Ankersmit et al. 2012; Hacker et al. 2009).

The role of DAMPs in the COPD pathogenesis has been currently discussed (Pouwels et al. 2014). The main function of these molecules is to regulate the activation of pattern recognition receptors. DAMPs can be divided into three broad categories: extracellular matrix components, molecules released during cellular stress, and secreted immunomodulatory proteins. The extracellular matrix components that may activate Toll-like receptors include fibronectin and hyaluronan. The stress-response molecules, including HSP, galectins, HMGB-1, and nucleic acids, which are released by cells on their dying, compose the second category. The last category includes immunomodulatory proteins, such as β-defensins and surfactant proteins, which are involved in a variety of biological processes, including host defenses and lung physiology (Tolle and Standifort 2013).

Heat shock proteins (HSPs) are chaperones that catalyze the proper folding of nascent proteins and refolding of denatured proteins. HSPs take part either in the renaturation or destruction of damaged proteins under stressful conditions such as heat, bacterial, or viral infections (Ritossa 1962). Under physiological conditions, synthesis of most HSPs is low. However, when the organism endures stress, such as heat shock and inflammation where protein damage is increased, HSPs are induced and expressed at high concentration (Feder and Hofmann 1999) A few reports estimated HSPs, especially HSP-27, in COPD patients. Hacker et al. (2009) have assessed HSP-27 in the serum of healthy non-smoking volunteers, smokers without COPD, patients with mild-to-moderate COPD, and patients with severe or very severe COPD. These authors report a significant difference between healthy controls and COPD, with a greater HSPs content in COPD. Similar results have been reported by Al Kayal et al. (2015) and Ünver et al. (2016). The findings on the HSPs of the present study add up to those in the serum above outlined, as we also found an association between BALF HSP-27 and pulmonary

function, in particular represented by the RV/TLC ratio; the association that has not been reported before. However, we found an inverse association between BALF HSP-27 and BODE index; the finding that is hardly reconcilable with the former. We presume the discrepancy between the two may be due to the fact that BODE index consists of a number of variables that may differentially affect HSP-27. On the other side, in contrast to the other authors, we failed to demonstrate an association between the serum content of HSP-27 and pulmonary function. That could be explicable by much advanced stages of COPD in the patients included into the study, compared with the studies above alluded to, as shown by the average FEV1 of 49% predicted.

Galectins are beta-galactoside-binding lectins that have a variety of physiological functions, including the control of intracellular trafficking of glycoproteins. These proteins, when released from damaged or dead cells, exhibit a pro-inflammatory function, which qualifies them as DAMPs (Liu et al. 2012). Gal-1 and Gal-3 are the most often studied galectins, have the strongest pro-inflammatory profile reported, and may contribute to the innate immune response involved in the pathogenesis of COPD (Pouwels et al. 2014). Gal-9 is a multifunctional protein with central participation in a number of cellular processes. However, how Gal-9 and its isoforms/splice variants are regulated at the expression, polyadenylation, posttranslational modification, and secretory levels is by far unclear (John and Mishra 2016). Pilette et al. (2007) have demonstrated that Gal-1 expression increases in the epithelial cells of small airways of smokers, compared with non-smokers and COPD patients; yet GAL-1 remains still significantly higher in COPD than the level in non-smokers. Those authors investigated eight COPD patients undergoing lung transplantation, six smokers and nine non-smokers undergoing lung surgery for a solitary peripheral lung tumor, but they failed to assess Gal-1 in the BALF and serum or the association between the galectins and clinical variables. In the present study, BALF Gal-1 positively correlated with the arterial PaO_2 and BODE index. This result indirectly confirms the hypothesis that increased lung Gal-1 content may be connected with COPD progression and poor oxygenation. Also, Gal-1 may be involved with systemic inflammation. An inverse association between the serum Gal-1 and oxygenation (PaO_2 and SaO_2), we noted, lends support to this speculation. There are just few studies concerning Gal-9 and COPD in the available literature. Vega-Carrascal et al. (2011) have detected Gal-9 in BALF taken from COPD patients, but they failed to relate it with clinical variables. Horio et al. (2017) have demonstrated that Gal-1 prevents the development of inflammation and emphysema in mice. However, the role of Gal-9 in the COPD inflammatory process is unclear. In the present study, we detected Gal-9 in the BALF and serum, with the association being found between Gal-9 and TLC in the serum only. Further studies are needed to explain the clinical meaning of this finding.

High mobility group box 1 (HMGB-1) is a member of non-histone chromosomal DNA-binding proteins (Anggayasti et al. 2017). The protein is involved with a number of DNA activities, such as DNA replication, repair, recombination, transcription, and in genomic stability. It also plays a significant role outside the cell in inflammation and immunity, as a mediator for cell growth, proliferation, and death (Gangemi et al. 2015; Kang et al. 2014). Hou et al. (2011) have evaluated HMGB1 in sputum and plasma in COPD, asthma, and in control subjects. They demonstrate the sputum HMGB1 content higher in asthma and COPD patients, compared with control subjects, and higher in COPD compared with asthma patients. Also, HMGB1 content in plasma and sputum show an inverse correlation with lung function variables in all subjects investigated in that study. On the other hand, Iwamoto et al. (2014) have conducted a four-year-long study in 32 non-smokers, 212 smokers without COPD, and 51 smokers with COPD. Those authors fail to show any difference between plasma HMGB1 content among the three groups of patients or any appreciable correlation between plasma HMGB1 content and pulmonary function. A study of Sobh et al. (2017) performed in 36 COPD patients and 39 healthy

volunteers show a correlation between plasma HMGB1 and matrix metalloproteinase 9 (MMP-9) and pulmonary function in COPD. Plasma HMGB-1 also positively correlates with the MMR dyspnea score and BODE index and COPD stage, and inversely with PaO_2, FEV1, FVC, and FEF25-75, and with 6MWD. In the present study, we detected HMGB1 in the BALF and serum of COPD patients, but the findings only fragmentally correspond to those previous studies as we demonstrate just an inverse association between the serum HMGB1 and SaO_2. This finding, however, points to a link between the HMGB1 released into the serum and poor oxygenation in COPD patients.

Surfactant protein A (SP-A) belongs to a family of proteins known as collectins. The protein was first identified in pulmonary surfactant (Pattle 1955). It is essential for pulmonary function as it reduces alveolar surface tension, plays a crucial role in innate immune responses, and in lung homeostasis (Nathan et al. 2016). Studies concerning the SP-A alterations in COPD are scarce. Kobayashi et al. (2008) have investigated the serum content of SP-A in 929 pulmonary patients (255 current smokers, 242 ex-smokers, and 432 never-smokers). SP-A was significantly higher in current smokers than in never- or ex-smokers, and significantly higher in the COPD smoking group. The protein positively associated with the intensity of smoking, and inversely with FEV1/FVC ratio, which makes it a potential marker of lung damage induced by smoking. The present findings are in line with those results in that we found the associations between serums SP-A content, smoking history, and pulmonary function.

5 Conclusions

The current study demonstrates that DAMPs occur in both lung microenvironment and serum in COPD patients. These molecules, except SP-A, do not seem affected by smoking history, but are involved in the local and systemic inflammatory process in COPD. Interestingly, not all of the DAMPs investigated in the study appeared involved with the lung damage and clinical variables featuring COPD. Therefore, further exploration of DAMPs influence on COPD progression is required, using alternative study designs, to settle their exact role in the disease, particularly in the accompanying lung inflammatory processes.

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MicroRNA-9 and Cell Proliferation in Lipopolysaccharide and Dexamethasone-Treated Naïve and Desialylated A549 Cells Grown in Cigarette Smoke Conditioned Medium

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Abstract

In this study we assessed microRNA-9 (miR-9) levels (RT-PCR) and cell proliferation (flow cytometry) in naïve and desialylated human alveolar epithelial cells (A549 cells), grown for 24 h in cigarette smoke-conditioned medium. Cells were additionally treated with lipopolysaccharide (LPS) and/or dexamethasone. Proliferation positively correlated with miR-9 levels in both naïve and desialylated cells. Cigarette smoke decreased miR-9 levels in both cell types by about three-fold but there was no significant correlation between both parameters. Dexamethasone was without substantial effect on cigarette smoke-induced changes in proliferation of naïve cells, but some normalization was observed in desialylated cells. Dexamethasone increased miR-9 levels in both cell types grown in cigarette smoke-medium but the effect was stronger in desialylated cells. LPS increased cell proliferation and miR-9 by more than six-fold only in naïve cells, while correlation coefficient for both parameters in cigarette smoke-LPS group was 0.41. Herein we identify miR-9 as the cigarette smoke (decrease) and LPS-responsive but dexamethasone-unresponsive microRNA. It is possible that increased miR-9 levels in naïve A549 cells treated with LPS may be related to the activation of Toll-like receptor 4. Moreover, differences in cell response (both miR-9 and proliferation) to dexamethasone in naïve and desialylated cells may point to non-genomic dexamethasone effects.

Keywords

Alveolar epithelial cells · Cell proliferation · Cigarette smoke · Dexamethasone · Lipopolysaccharide · microRNA-9 · Sialic acid

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1 Introduction

Long-term exposure to cigarette smoke (CS) increases the risk for lung cancer but the mechanisms of CS-induced airway inflammation and biochemical pathways related to malignant transformation of respiratory system cells are unknown. In recent years, the discovery of microRNAs has made it evident that these molecules have widespread control of the expression of proteins involved in inflammatory signaling and play an essential role in the initiation and development of cancer (Xue et al. 2014). CS-exposed animals have an extensive repression of a number of miRNAs (Cohen et al. 2016). In the lungs of rats exposed for 4 weeks to CS there is a widespread but reversible down-regulation of 126 miRNAs (Izzotti et al. 2009). Longer exposure to CS may produce persistent microRNA repression with progressive development of cancer (Izzotti et al. 2011).

In the present study, we assessed microRNA-9 (miR-9) levels and cell proliferation in naïve and desialylated human alveolar epithelial cells (A549 cell line), grown for 24 h in CS-conditioned medium, supplemented with lipopolysaccharide (LPS) or dexamethasone (DEX). Our experiments were performed on naïve A549 cells and on A549 cells with depleted cell membrane sialic acid. Cell surface sialoproteins are relevant not only to metastatic potential of cancer cells but are also important to the host resistance to viral and bacterial infections and to immune response (Lundahl et al. 2017). Our earlier data have evidenced increased expression of the sialic acid-binding receptors Siglec-5/14 in chronic obstructive pulmonary disease (COPD) patients receiving corticosteroids (Wielgat et al. 2015). Similar changes involving Siglec 8 have been described in bronchial asthma (Kiwamoto et al. 2013) and Siglec 14 in COPD exacerbation (Ishii et al. 2017). Moreover, increased Siglec-8 (Mroz et al. 2013) in sputum and Siglec-9 levels in alveolar and peripheral blood neutrophils have been found in COPD patients (Zeng et al. 2017).

Our aim was to quantify miR-9 in naïve and desialylated A549 cells grown in CS-conditioned medium under pro-inflammatory (LPS) and anti-inflammatory (DEX) stimuli and to analyze changes in miR-9 in relation to cell proliferation. MiR-9 expression is especially interesting since apart from its possible suppressive role in cancer, miR-9 may inversely affect DEX sensitivity in an experimental model of steroid-resistant airway hyperresponsiveness (Li et al. 2015, 2017).

2 Methods

2.1 Cell Culture

A549 (ATCC® CCL185™) cells grown in ATCC-formulated F12 K medium supplemented with 10% fetal bovine serum (FBS) and THP1 cells (ATCC® TIB202™) grown in ATCC-formulated RPMI 1640 medium, supplemented with 2-mercaptoethanol to a final concentration of 0.05 mM and with FBS to a final concentration of 10% were used in this study. Cells were maintained in 37 °C in an incubator in a humidified atmosphere containing 5% CO_2. For particular experiments cells were plated out onto 6 well plates.

2.2 Preparation of CS-Conditioned Media and Cell Treatment

CS-conditioned medium was prepared using full-strength Red Marlboro cigarettes (Phillip Morris; Cracow, Poland) containing about 8.0 mg of tar, 0.6 mg of nicotine, and 9.0 mg of carbon monoxide *per* cigarette. To prepare smoke-conditioned media cigarette filters were removed and smoke was passed through culture media (4 cigarettes/100 ml of medium) using a low pressure vacuum pump. Freshly prepared CS media were diluted with standard media to obtain 30 μM nitrate/nitrite content in each batch (colorimetric reaction with Griess reagent). CS-conditioned media were subsequently filtered using 0.22 μm filters and were

applied to cell culture. Naive or desialylated, preincubated with neuraminidase (100 U/ml) for 24 h, A549 cells were grown in CS-conditioned medium for 24 h. In some experiments cells were additionally treated with LPS (1 μg/ml) or DEX (10^{-5} M) for 24 h.

2.3 miR-9 Levels

To quantify miR-9, reverse transcription and quantitative PCR (qPCR) were performed using the TaqMan™ microRNA assay kit (cat. #604366596) (Thermo Fisher Scientific; Waltham, MA) following the producer's protocol. U6 small RNA was used as a reference. The Thermo Fisher Scientific catalog numbers for miR-9 and U6 were: 4427975, assay ID: 000583, assay name: hsa-miR-9 and 4427975, assay ID-001093, assay name: RNU6B, respectively. MiR-9 expression was calculated with ΔΔCt method and was shown as fold changes.

2.4 Cell Proliferation

Cell proliferation was quantified in flow cytometry (Epics XL flow cytometer; Coulter Electronics, High Wycombe, UK) using propidium iodide DNA staining and cell cycle analysis (Brown et al. 1996). Histograms of propidium iodide fluorescence distributions were quantified using MultiCycle software and cells were quantified by their relative distribution in the damaged-subdiploid ('early' G0/G1 cells), diploid (G0/G1 zone)-pre-DNA synthesis/resting, S-phase-DNA synthesis, and G2/M-post-DNA-synthesis/mitosis phases. Each histogram was derived from analysis of 5000 cells and five samples were analyzed in each group. Proliferating cells were quantified as S+G2/M cells.

2.5 Statistical Analysis

Results were expressed as means ±SD of 3–5 assays run in triplicate. One-way or two-way ANOVA, followed by Bonferroni *post-hoc* test for selected pairs of data, was used to analyze statistical differences. The relationship between mir-9 expression and cell proliferation was examined using a linear regression analysis. A p-value < 0.05 defined statistically significant differences. The analysis was performed with a commercial statistical package of Statistica v6.0 (Statsoft; Cracow, Poland).

3 Results

Table 1 and Fig. 1 show the proliferation index (S + G2M cell fractions; Fig. 1a) and miR-9 expression (Fig. 1b) in naive and desialylated

Table 1 Alterations in cell proliferation and miR-9 level in naïve and desialylated A549 cells grown for 24 h in cigarette smoke (CS)-conditioned medium, medium supplemented with lipopolysaccharide (LPS; 1 μg/mL) or dexamethasone (DEX; 10^{-5} M). Correlation coefficient (r) was calculated to assess relationship between cell proliferation and miR-9 levels in each group

	Naïve A549 cells			Desialylated A549 cells		
	Proliferation (S+G2/M cells; %)	miR-9 (fold-change)	Correlation Coefficient (r)	Proliferation (S+G2/M cells; %)	miR-9 (fold-change)	Correlation Coefficient (r)
Control	23 ± 5	1 ± 0.2	0.62*	28 ± 8	1.1 ± 0.2	0.44
CS	$8 \pm 5^{**}$	$-2.7 \pm 0.3^{**}$	0.21	$9 \pm 4^{**}$	$-3.3 \pm 0.7^{**}$	0.38
LPS	$42 \pm 8^{**}$	$6.1 \pm 1.0^{**}$	0.47	$18 \pm 6^{*\#\#}$	$1.9 \pm 0.4^{**\#\#}$	−0.33
DEX	$47 \pm 9^{**}$	1.3 ± 0.5	−0.18	$23 \pm 7^{\#\#}$	1.7 ± 0.5	0.27
CS + LPS	$11 \pm 4^{**\#\#}$	$-2.9 \pm 0.5^{**}$	0.41	$12 \pm 6^{**}$	$1.2 \pm 0.6^{\#\#\wedge\wedge}$	0.17
CS + DEX	$10 \pm 5^{**\#\#}$	$3.3 \pm 0.6^{**\#\#}$	−0.28	$19 \pm 7^{*\#\#\wedge}$	$10.2 \pm 1.5^{**\#\#\wedge\wedge}$	−0.29

$^{*}p < 0.5$; $^{**}p < 0.01$ for comparisons with the corresponding control cells; $^{\#\#}p < 0.01$ for comparisons with naïve cells subject to the same treatment; $^{\wedge}p < 0.05$; $^{\wedge\wedge}p < 0.01$ for comparisons with corresponding cells grown in CS-conditioned media

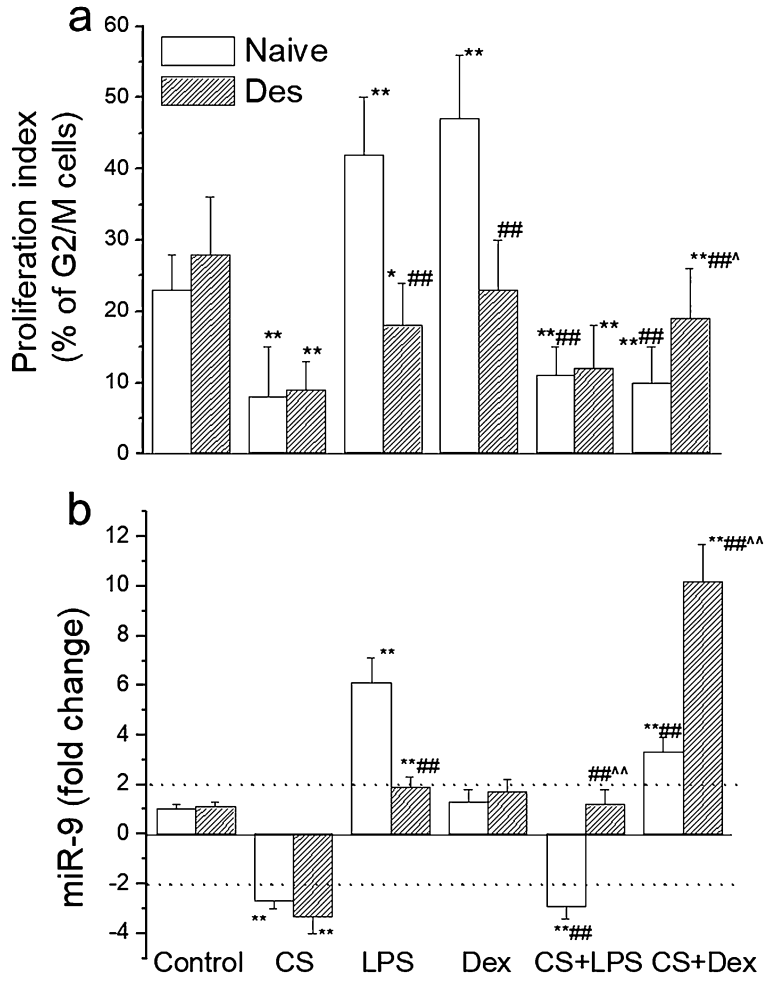

Fig. 1 Alterations in cell proliferation (Panel a) and microRNA-9 (miR-9) expression (Panel b) in naïve and desialylated A549 cells grown for 24 h in cigarette smoke (CS)-conditioned medium supplemented with lipopolysaccharide (LPS; 1 μg/ml) or dexamethasone (Dex; 10^{-5} M). Dotted lines in Panel b encompass two-fold changes of gene expression, both decreases and increases, which are considered statistically significant
$^{*}p < 0.05$; $^{**}p < 0.01$ for comparisons with the corresponding control cells
$^{\#\#}p < 0.01$ for comparisons with the naïve cells subject to the same treatment
$^{\wedge}p < 0.05$; $^{\wedge\wedge}p < 0.01$ for comparisons with the corresponding cells grown in cigarette smoke (CS)-conditioned media

A549 cells grown for 24 h in CS-conditioned medium or CS medium supplemented with LPS or DEX. Moreover, the correlation coefficient (r) was calculated to assess the relationship between cell proliferation and miR-9 expression in each subgroup.

Cell proliferation positively correlated with miR-9 levels only in the control groups, reaching an *r*-value of 0.62 in naïve cells and *r* of 0.44 in untreated, desialylated cells. CS significantly decreased fractions of proliferating cells. The antiproliferating effect of CS was similar in naïve and desialylated cells (a decrease by 75% and 87%, respectively; $p < 0.01$). Both DEX and LPS added to normal culture medium significantly increased fractions of proliferating cells ($p < 0.01$), but it was the case only in naïve cells while *r* value in the CS + LPS group was 0.41.

In desialylated cells, DEX was without a significant effect on the proliferation rate, while LPS decreased proliferation index ($p < 0.05$). In the LPS-treated naïve cells, both epithelial cell proliferation and miR-9 expression were positively correlated ($r = 0.47$), which was not observed in desialylated cells. LPS did not affect a decreased cell proliferation in CS-exposed cells, irrespective of cell sialylation patterns In the cells grown in CS-conditioned media with DEX, there was no

pro-proliferating effect in naïve cells, while some normalization of cell growth was observed in desialylated cells (CS *vs*. CS + DEX; $p < 0.05$).

CS decreased the expression of miR-9 in naïve and desialylated cells by more than two-fold, while LPS increased miR-9 by more than six-fold ($p < 0.01$), but only in naïve cells. DEX was without a substantial effect on miR-9 in both cell types, but when naïve cells were treated with CS and DEX, miR-9 expression was elevated ($p < 0.01$) in both cells types. Interestingly, LPS normalized a decreased miR-9 level in the CS-treated desialylated A549 cells, but not in naïve cells.

4 Discussion

Epithelial cells of the respiratory tract are the main target of CS. Chronic exposure to CS produces epithelial cell damage, chronic inflammation, functional and structural changes in the airway tissue, and lung cancer (Sarma and Ward 2011). In this study, we assessed changes in, and mutual relationship between, human alveolar epithelial cell proliferation and miR-9 expression in naïve and desialylated cells, grown for 24 h in CS-conditioned medium and medium supplemented with LPS or DEX. MiR-9 is especially relevant for airway inflammation because it may be involved not only in lung cancer (Li et al. 2017), but also in reduced responsiveness to corticosteroids (Li et al. 2015), which is the main barrier against a successful therapy in smoking patients with asthma or COPD. In the control naïve and desialylated cells, cell proliferation positively correlated with miR-9 levels. CS induced a decrease in proliferating cell fraction, irrespective of cell membrane sialylation due most probably to significant toxicity (Holownia et al. 2015). CS contains highly reactive mutagenic compounds and alterations in the level of miRNA may be an early event in CS-induced carcinogenesis. In this study, CS decreased the level of miR-9 in naïve and desialylated cells, but the correlation between alveolar epithelial cell proliferation and miR-9 expression was low. It has been previously reported that the CS-induced down-regulation of miRNA may cause oncogene activation and an increase in cell proliferation, which can lead to carcinogenesis (Izzotti et al. 2011; Schembri et al. 2009). Downregulation of miRNAs in the bronchial airway epithelium due to CS exposure is inversely related to miRNAs biochemical targets of carcinogenesis. Similar changes have been described in alveolar macrophages in heavy smokers (Graff et al. 2012). The role of miR-9 is less clear in oncogenesis. miR-9 is considered a pro-oncogene in breast cancer (Gang et al. 2017), but not in ovarian cancer (Guo et al. 2009) where it plays the opposing suppressing role. Recently, it has been shown that miR-9-5p promoted cell invasion, migration, and proliferation in non-small cell lung cancer through the repression of transforming growth factor (TGF) beta type 2 (TGFBR2) receptor that regulates cell proliferation and differentiation, immunosuppression, and carcinogenesis (Gang et al. 2017).

In this study, CS decreased cell proliferation irrespective of cell membrane sialylation, while LPS-mediated increase in cell growth was lower in desialylated cells. It is possible that desialylation may affect a LPS-dependent TL4 receptor. It has been shown that in pulmonary macrophages exposed to LPS, miR-9 is increased, and inhibition of miR-9 restores steroid sensitivity (Li et al. 2015). In the present study, cells treated with CS + DEX had a significantly higher level of miR-9 than the CS alone-treated cells. However, cell proliferation was partly normalized by DEX only in desialylated cells, which may point to some indefinite cell membrane effects.

We herein report the changes induced by CS, LPS, and DEX in naïve and desialylated human epithelial cell line and identify miR-9 as the CS (decrease) and LPS-responsive but DEX-unresponsive microRNA. According to the published data, downregulation of miR-9 by CS may be associated with lung cancer (Tibaldi et al. 2015), but the exact underlying mechanism is unclear. It is possible that increased miR-9 levels in naïve A549 cells treated

with LPS may be related to the activation of Toll-like receptor 4 (TLR4) in the epithelial cell membrane. Moreover, differences in the cellular response, in both miR-9 and proliferation, to DEX in naïve and desialylated cells may point to the possible role of non-genomic DEX effects, but more data are needed to define the role of microRNA in cigarette smoke-induced inflammation and carcinogenesis.

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

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COPD Course and Comorbidities: Are There Gender Differences?

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Abstract

The prevalence of chronic obstructive pulmonary disease (COPD) has increased more rapidly in women than in men during the past two decades. Clinical presentation, comorbidities and prognosis may differ between genders and may influence management decisions. The influence of gender on COPD expression has not been clearly explained to date. Thus, the aim of this study was to evaluate significant differences between women and men suffering from COPD, regarding clinical presentation, pulmonary function test results, comorbidities, and prognosis. We prospectively recruited 470 patients with stable COPD with a history of smoking (152 women, 318 men, mean age 65.5 ± 8.8 *vs*. 66.6 ± 9.4 years, respectively). Comorbidities and exacerbations were recorded. Spirometry, body plethysmography, carbon monoxide diffusing capacity and 6-min walk tests were performed. The BODE prognostic score was also calculated. We found that women smoked less in comparison to men (30.4 *vs*. 41.9 pack-years, $p < 0.05$), showed more exacerbations (2.5 *vs*. 1.7, $p = 0.01$), higher forced expiratory volume in 1 s (FEV1%predicted), and increased residual volume/total lung capacity (RV/%TLC), but they had the same intensity of dyspnea. Women showed fewer comorbidities, on average, *per* patient (5.4 *vs*. 6.4, $p = 0.002$), but had a higher prevalence of at least seven comorbidities *per* patient (48.7% of women vs. 33.0% of men, $p < 0.05$). Women also had a significantly worse prognosis (4.6 *vs*. 3.1 BODE score, $p < 0.05$) that correlated with the number of comorbidities ($r = 0.33$, $p < 0.01$). In conclusion, this study strongly supports the existence of different gender phenotypes in COPD, especially regarding exacerbations, comorbidities, and prognosis. The gender difference may indicate a need for a targeted assessment and management of COPD in women and men.

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Keywords
Comorbid conditions · COPD · Exacerbations · Gender differences · Prognosis · Pulmonary function · Sex phenotypes · Smoking

1 Introduction

Chronic obstructive pulmonary disease (COPD) is one of the most common diseases worldwide and is typically characterized by progressive, persistent airflow limitation associated with enhanced chronic inflammatory responses in the airways and lungs to noxious particles or gases (Decramer et al. 2013). The prevalence of COPD is high and has increased more rapidly in women than men during the past two decades (WHO 2008). COPD is currently the fourth leading cause of death worldwide. According to WHO's predictions, COPD will become the third leading cause of death by 2030. Thus, COPD constitutes an important public health challenge leading to economic and social burdens (Teo et al. 2012). The course of COPD varies depending on the respiratory system pathologies and dysfunction of other organs. It is known that patients with COPD have comorbidities that influence the course of the disease, quality of life, and prognosis (Mannino et al. 2008; Sin et al. 2006; Soriano et al. 2005). The severity of comorbid conditions and their impact on health status varies not only between patients but also in the same patient over time. Efficient treatment of comorbidities is associated with a more stable course of COPD and better outcomes. Therefore, patients with COPD require comprehensive, specialized care. The influence of gender on the expression of COPD, which may influence the management strategy, has not been clearly explained to date. Therefore, the aim of the current study was to evaluate differences between women and men suffering from COPD regarding clinical presentation, pulmonary function test results, comorbidities, and prognosis.

2 Methods

The study was approved by the institutional Ethics Committee. We prospectively recruited COPD patients attending pulmonary outpatient offices and clinics of five pulmonology departments in Poland from 2009 to 2012. A total of 470 patients were included in the study (152 women, 318 men). The inclusion criteria consisted of a diagnosis of COPD according to the guidelines of the 2009 Global Initiative for Chronic Obstructive Lung Disease - GOLD (Rodriguez-Roisin et al. 2009), with a forced expiratory volume in 1 s (FEV1) to forced vital capacity (FVC) ratio of <70% after inhalation of 400 μg of salbutamol and at least a 10 pack-years history of smoking. Patients were clinically stable (no exacerbation for at least 12 weeks) at the time of inclusion. Characteristics of the study group are shown in Table 1. COPD severity was assessed according to the Global Strategy for the Diagnosis guidelines above mentioned and was distributed as follows: I-7%, II-45%, III-36%, and IV-12% of the patients.

The presence of comorbidities was evaluated based on a questionnaire prepared for this study.

Table 1 Characteristics of COPD patients

	Mean ± SD	Median
Age (years)	66.2 ± 9.3	66.0
BMI (kg/m^2)	28.0 ± 6.4	27.1
Pack-years	37.8 ± 21.4	38.0
Last smoked (years)	8.4 ± 10.0	5.0
Passive smoking (years)	28.6 ± 16.9	29.0
COPD diagnosis (years)	7.7 ± 6.8	6.0
No. of exacerbations/2 years	2.0 ± 2.8	1.0
FEV1 post (% pred)	52.5 ± 18.4	51.7
TLC (% pred)	119.3 ± 22.4	117.7
RV (% pred)	177.5 ± 55.8	169.5
RV/%TLC	60.0 ± 10.0	60.0
DLCO (% pred)	64.3 ± 27.5	63.9
6MWD (m)	384.4 ± 129.9	390.0
6MWD (% pred)	77.9 ± 26.1	80.4

BMI body mass index, *FEV1* forced expiratory volume in 1 s, *TLC* total lung capacity, *RV* residual volume, *DLCO* diffusing capacity of carbon monoxide, *6MWD* 6-min walk distance

Data regarding medical history, clinical interviews, physical examination as well as previous and current test results were taken into account. Spirometry, body plethysmography, and carbon monoxide diffusing capacity (DLCO) tests were performed according to European Respiratory Society/American Thoracic Society (ERS/ATS) guidelines using MasterScreen Body device (Jaeger; Hoechberg, Germany) (MacIntyre et al. 2005; Miller et al. 2005; Wanger et al. 2005). Dyspnea was estimated with the modified Medical Research Council (mMRC) scale, and exercise capacity was tested with the 6-min walk test (6MWT) following ATS criteria (ATS 2002). Body mass index (BMI) was calculated and the BODE score as a predictor of the risk of death in accordance with recommendations by Celli et al. (2004). Arterial blood gases were measured at rest. Exacerbations were defined according to the GOLD criteria as events in the natural course of the disease characterized by a change in baseline dyspnea, cough and/or sputum that exceeded normal daily variations, were acute in onset and warranted a change in regular medications (Rodriguez-Roisin et al. 2009). A period of 2 years preceding study inclusion was taken into account for the recording of exacerbations.

Data are presented as means and medians for continuous variables and as numbers and percentages for categorical variables. Data were compared using Student's *t*-test when the test statistics followed – normal distribution or in case of a skewed distribution with the Mann-Whitney *U*-test for continuous variables and the Fisher exact test for categorical variables. One-way analysis of variance (ANOVA) on ranked variables (Kruskal-Wallis test by rank) was used for comparing two or more independent samples. Spearman's rank correlation coefficient was used to assess the relationship between two variables. A p-value of < 0.05 was considered statistically significant. Statistical analysis was performed using a commercial Statistica package (StatSoft; Cracow, Poland).

3 Results

There was no difference in age between the women and men included in the study (65.5 ± 8.8 *vs.* 66.6 ± 9.4 years, respectively, $p > 0.05$). Women showed a slightly, but significantly, lower BMI (25.3 ± 9.3 *vs.* 26.6 ± 9.7 kg/m^2, $p < 0.05$), fewer pack-years of smoking (30.4 ± 19.4 *vs.* 41.9 ± 23.8; $p < 0.05$) and a shorter time since quitting than men (6.8 ± 8.6 vs. 9.1 ± 10.4 years; $p < 0.05$). There was a lower prevalence of women expectorating sputum than men (59.5 *vs.* 76.3%; $p = 0.0003$), but they had the same intensity of dyspnea as men as measured by the mMRC scale ($p = 0.66$). Women also had a shorter COPD disease duration than men (6.6 ± 6.0 *vs.* 8.2 ± 7.2 years; $p < 0.05$).

Women had significantly more exacerbations than men (2.5 ± 3.0 *vs.* 1.7 ± 2.7 respectively; $p = 0.01$) and more frequently developed at least two or four exacerbations in the past 2 years in comparison to men ($p = 0.03$) (Fig. 1). The number of exacerbations correlated significantly with the number of comorbidities in both women and men. However, for women, this relationship was slightly stronger (women $r = 0.29$, $p < 0.01$; men $r = 0.22$, $p < 0.01$).

Taking pulmonary function results into account, women demonstrated significantly

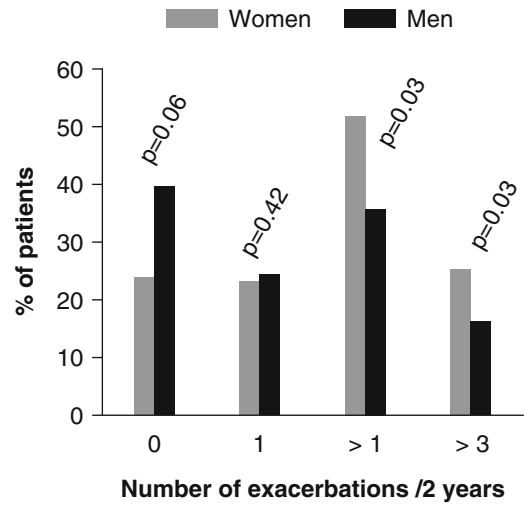

Fig. 1 Gender differences in the prevalence of COPD exacerbations during the 2 years prior to inclusion

higher values for FEV1, total lung capacity (TLC), residual volume (RV), expressed as % predicted (% pred), RV/%TLC, and a higher partial pressure of oxygen in arterial blood gases (PaO_2) than men (69.0 ± 12.2 *vs.* 65.7 ± 11.0 mmHg; $p < 0.05$). A comparison of characteristics by gender is shown in Table 2.

The most common comorbidities were systemic hypertension, peripheral edema, ischemic heart disease, varicose veins, movement problems, and cardiac arrhythmias. Women showed fewer comorbidities than men, on average, 5.4 ± 3.7 *vs.* 6.4 ± 3.2; $p = 0.002$, although there was a higher prevalence of females with at least seven comorbidities (48.7 *vs.* 33.0%, $p < 0.05$) (Fig. 2). There were also some significant differences in comorbidity types between genders (Fig. 3).

Table 2 Characteristics of COPD patients by gender

	Women	Men	p
Age (years)	65.5 ± 8.8	66.6 ± 9.4	0.213
BMI (kg/m^2)	25.3 ± 9.3	26.6 ± 9.7	0.040
Pack-years	30.4 ± 19.4	41.9 ± 23.8	<0.001
Last smoked (years)	6.8 ± 8.6	9.1 ± 10.4	0.038
Passive smoking (years)	26.1 ± 17.7	30.0 ± 16.4	0.209
COPD diagnosis (years)	6.6 ± 6.0	8.2 ± 7.2	0.040
No. of exacerbations/2 years	2.5 ± 3.0	1.7 ± 2.7	0.010
FEV1 post (% pred)	55.5 ± 19.4	51.0 ± 17.8	0.014
TLC (% pred)	128.4 ± 24.0	114.4 ± 19.8	<0.001
RV (% pred)	188.4 ± 58.7	171.6 ± 53.4	0.006
RV/%TLC	60.0 ± 10.0	56.0 ± 11.0	0.001
DLCO (% pred)	64.7 ± 26.0	64.1 ± 28.4	0.876
6MWD (m)	466.5 ± 62.8	392.1 ± 127.5	<0.001
6MWD (% pred)	79.2 ± 36.8	77.3 ± 25.1	>0.05
PaO_2 (mmHg)	69.0 ± 12.2	65.7 ± 11.0	0.040
BODE	4.6 ± 0.5	3.1 ± 2.5	<0.001
No. of comorbidities	5.4 ± 3.7	6.4 ± 3.2	0.002

Data are means ± SD. *BMI* body mass index, *FEV1* forced expiratory volume in 1 s, *TLC* total lung capacity, *RV* residual volume, *DLCO* diffusing capacity of carbon monoxide, *PaO_2* partial pressure of arterial oxygen, *BODE* prognostic index in COPD, *6MWD* 6-min walk distance

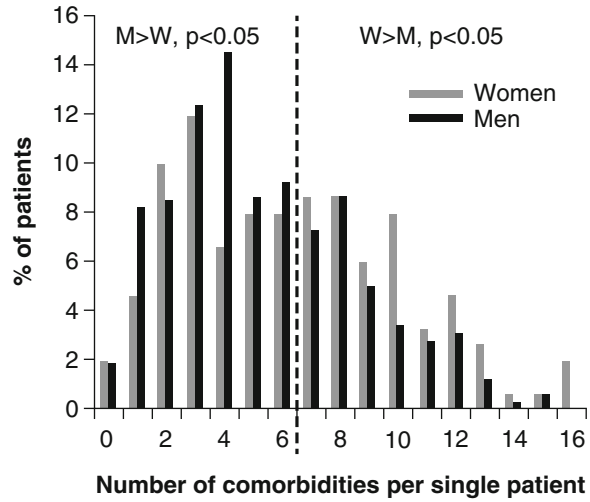

Fig. 2 Gender differences concerning comorbidities. Note a higher prevalence of at least seven comorbidities among women and fewer than seven among men; *W* women, *M* men

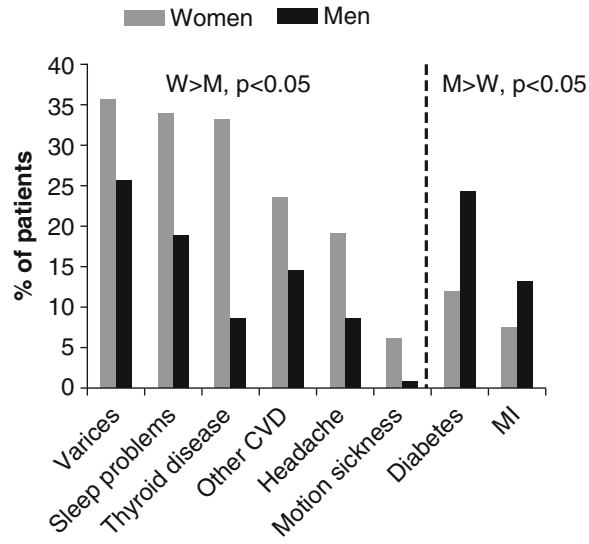

Fig. 3 Gender differences concerning comorbidities. CVD, cardiovascular diseases: valvular heart disease, aortic aneurysm, and cardiomyopathy, *MI* myocardial infarction, *W* women, *M* men

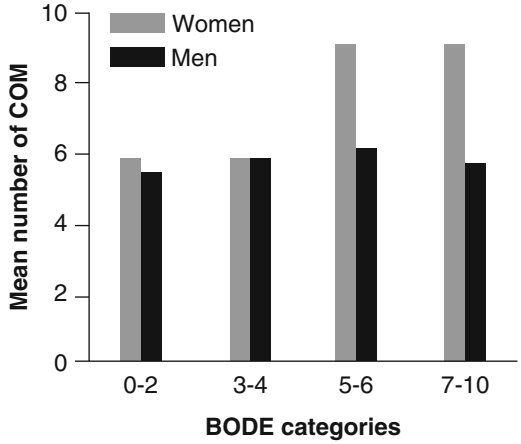

Fig. 4 Gender differences concerning comorbidities (COM) in relation to BODE score. Significant differences in the number of COM in BODE categories concerned only women ($p < 0.05$). Differences between women and men were only for BODE ≥ 5 ($p < 0.05$)

Women had significantly worse prognoses than men as measured by a higher BODE index score (4.6 ± 0.5 *vs*. 3.1 ± 2.5 points; $p < 0.05$). Women with a higher risk of death based on the BODE index (score ≥ 5) also had significantly more comorbidities than those with a lower risk of death (9.1 ± 3.8 *vs*. 5.8 ± 3.3; $p < 0.05$); there was no such relationship in men ($p = 0.77$). Among all patients with a BODE score of at least 5, women showed more comorbidities than men (9.1 ± 3.8 *vs*. 5.9 ± 3.5; $p < 0.05$) (Fig. 4). The number of comorbidities correlated positively with the BODE score in women ($r = 0.33$, $p < 0.01$) in contrast to men, for whom this dependence was not present ($r = 0.08$, $p = 0.17$).

4 Discussion

This multicenter study was the first to analyze a Polish population of COPD patients in relation to gender differences. The main findings were that women developed more exacerbations in comparison to men, had more advanced lung hyperinflation, and showed a higher prevalence of numerous comorbidities with a higher risk of death despite less exposure to tobacco.

4.1 Symptoms and Pulmonary Function

Symptoms are a good predictor of disease status, especially in men, as shown in the Euroscop study (Watson et al. 2006). According to a study conducted by Lange et al. (2012), patients in the B category of COPD classification who had more

advanced symptoms, predominantly dyspnea, show poorer survival than those who were less symptomatic but with lower FEV1. Women in our group reported sputum expectoration less frequently than men, in agreement with other studies. However, these women also had the same intensity of dyspnea according to the mMRC scale, which is different from the results of some previous research (Celli et al. 2011; Gershon et al. 2010; Cydulka et al. 2005; de Torres et al. 2005; Watson et al. 2004). Nevertheless, investigators in Spain recently achieved the same results as we did in the current study (Ancochea et al. 2013). Gender differences in clinical presentation are not clear. One possible explanation might be a difference between women and men in reporting of symptoms such as sputum production because of sociocultural influences. Phlegm expectoration can be embarrassing and may lead to underreporting among women (Camp and Goring 2007). We did not use the COPD Assessment Test (CAT) which could measure the intensity of symptoms more accurately and objectively, because it was not implemented into clinical practice at the time of this study. Overall, these results suggest that the bias in symptom reporting by women could result in misclassification of COPD category and, consequently, inadequate treatment. In women, a simple scale such as the mMRC might be insufficient to assess disease intensity. Thus, more accurate questionnaires (e.g., CAT) and tests are needed for a more reliable assessment. A precise knowledge about symptom intensity in COPD is crucial for optimal therapeutic decision-making.

Women in our group showed more advanced hyperinflation expressed by a higher RV % pred and an increased RV/%TLC ratio, the functional signs of emphysema which favor breathlessness, despite a higher FEV1% pred than that in men. Previous studies have shown that men with COPD predominantly experienced emphysema, while women experienced chronic bronchitis. However, this tendency has recently changed. There is now evidence that more women than men in the US have been diagnosed with emphysema (CDC 2011; Burrows et al. 1987). Our present results confirm this observation.

4.2 Exacerbations

The two-year period preceding study inclusion was taken into account for exacerbation recording to better assess the course of COPD. Women in our study developed more exacerbations of COPD than did men, as also has been found in other studies (Celli et al. 2011, Agusti et al. 2010). COPD exacerbations rapidly worsen both the patient's condition and the course of the disease. Therefore, prevention of exacerbations is a crucial goal of COPD management, which may be especially important for women as shown by the results of this study.

4.3 Comorbidities

Patients with COPD often have coexisting medical conditions (Sievi et al. 2015). We observed significant differences in the number of comorbidities between women and men when we analyzed the whole study group. On average, men had more comorbidities than women. Nevertheless, we noticed differences in the distribution of comorbidities, with more women showing more numerous comorbidities *per* patient. Although gender differences in comorbidities remain underexamined, our results show a different pattern of comorbidities between women and men, similar to the findings of the ECLIPSE study (Agusti et al. 2010). In particular, men were found to suffer more frequently from ischemic heart disease and diabetes, whereas women suffered more frequently from varicose veins, sleep problems, thyroid diseases, other cardiovascular diseases (valvular heart disease, aortic aneurysm, or cardiomyopathy), headache, and motion sickness. Numerous comorbid diseases in women may contribute to their worse COPD course and

prognosis. Therefore, women with numerous comorbidities should be carefully monitored and managed.

4.4 Prognosis

According to previous research, women develop similar or even more severe COPD at younger ages than men with lower levels of cigarette exposure (Martinez et al. 2007; Murray and Lopez 1997). Gan et al. (2006) have found that female smokers had a more rapid decline in FEV1 than male smokers. Comparison of the results of the NHANES I and NHANES III studies shows that among patients with COPD, there is a smaller decline in mortality rate in women than in men (Eisner et al. 2010). We noticed a worse prognosis in women than in men regarding BODE index scores, and women with higher BODE scores had significantly more comorbid diseases, an additional risk factor for poor outcomes. In contrast, there was no such relationship in men. This finding suggests that a BODE score of at least five, with numerous comorbidities, confers a significantly worse prognosis for women than men, necessitating more attention for care of these women. According to Jones et al. (2011) patients with COPD and more than three comorbidities show higher CAT scores, thus indicating a greater impairment of health status. In another study, the risk of rehospitalization was higher among patients with lower health status (Gudmundsson et al. 2006).

4.5 Limitations

The women in our study had a higher FEV1% pred, suffered from COPD for a shorter time, smoked at later ages and fewer pack-years in comparison to men, but there was no difference in age between the women and men. These findings suggest that women may develop more advanced obstruction and disease severity at a later age than do men. Moreover, the vast majority of never smokers suffering from COPD in previous studies were women (Lamprecht et al. 2011, Chen et al. 2000), which indicates that women may be more susceptible to nonsmoking-related factors. The increased susceptibility to cigarette smoke and other risk factors in women may result from gender-specific genetic predisposition, smaller lungs and airways, different inhalation techniques, cigarette preferences, and hormonal features (Shambhu et al. 2013). Other studies have shown that women quit smoking less frequently and have a lower rate of successful smoking cessation than men (Satcher et al. 2001). We did not assess the prevalence of depression, which was shown in other studies to influence the course of COPD and to be more frequent in women (Raherison et al. 2014). Thus, there still remain unknown aspects of gender differences in COPD that require further exploration (Han et al. 2007).

4.6 Summary

In summary, the course of COPD differs based on gender. Our results show that women had more frequent exacerbations, more advanced hyperinflation, and higher BODE scores, which come to worse prognosis, than men despite less tobacco exposure. Further, a higher prevalence of numerous comorbidities in women with COPD correlated with a higher risk of death as measured by BODE score. The results of this study strongly suggest the existence of different gender phenotypes in COPD, which supports the need for targeted assessment and management of COPD in women and men. Further research is expected to clarify the pathological mechanisms of gender differences.

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

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Post-traumatic Stress Disorder: A Review of Therapeutic Role of Meditation Interventions

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Abstract

This review is an attempt to provide a comprehensive view of post-traumatic-stress disorder (PTSD) and its therapy, focusing on the use of meditation interventions. PTSD is a multimodal psycho-physiological-behavioral disorder, which calls for the potential usefulness of spiritual therapy. Recent times witness a substantial scientific interest in an alternative mind-to-body psychobehavioral therapy; the exemplary of which is meditation. Meditation is a form of mental exercise that has an extensive, albeit still mostly empiric, therapeutic value. Meditation steadily gains an increasing popularity as a psychobehavioral adjunct to therapy in many areas of medicine and psychology. While the review does not provide a final or conclusive answer on the use of meditation in PTSD treatment we believe the available empirical evidence demonstrates that meditation is associated with overall reduction in PTSD symptoms, and it improves mental and somatic quality of life of PTSD patients. Therefore, studies give a clear cue for a trial of meditation-associated techniques as an adjunct to pharmacotherapy or standalone treatment in otherwise resistant cases of the disease.

Keywords

Breathing · Meditation · Mindfulness of breathing · Posttraumatic stress disorder · Psychobehavioral therapy

1 Post-traumatic Stress Disorder

Post-traumatic stress disorder (PTSD) is a clinical syndrome that may develop following extreme traumatic stress. It is an important, albeit relatively uncommon, consequence of exposure to traumatic events, presumably the result of life threats and conditioned fear (Greenberg et al. 2015; Ramage et al. 2016). PTSD is recently defined by four categories of socio-psychological symptoms (DSM-V 2013): (1) intrusion that encompasses re-experiencing the traumatic event through intrusive memories, flashbacks, nightmares, and physiological responses similar to those when the traumatic event occurred; (2) avoidance that encompasses mind-numbing occurrences, such as avoiding situations and people reminding of past trauma, amnesia for trauma-related information, loss of interest in activities, social and emotional detachment, emotional

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numbing especially for feelings associated with intimacy, and nihilistic feelings of the future; (3) changes in arousal manifested by angry outbursts, sleep problems, startle responses, and hypervigilance; and (4) mood and cognition disorders consisting of difficulty to cope by feeling down and hopeless, dysphoric mood, problems with judgment, reasoning, and emotion perception, as well as with focusing attention on a task completion.

PTSD is a global health issue (Jindani et al. 2015; Ramchand et al. 2015). The disorder develops in approximately 20% of people exposed to a traumatic event (Freedman et al. 2015). It is more prevalent in females than males: typically about twice the rate (Jaycox et al. 2004; Kessler et al. 1995). It affects about 8% of the general US population at some point during their lifetime (Gates et al. 2012). Risk factors for PTSD in adults vary across studies. The three factors identified as having relatively uniform effects are the following: (1) preexisting psychiatric disorders; (2) family history of such disorders; and (3) childhood trauma (Breslau 2002). The lifetime prevalence in the US female population is more than 10% (Kessler et al. 1995). The prevalence rate of lifetime PTSD in Canada is estimated at 9.2%, with a rate of current (one-month) PTSD of 2.4% (Van Ameringen et al. 2008). According to the 2013 Canadian Forces Mental Health Survey, 5.3% of soldiers report experiencing PTSD at some point of service (Zamorski et al. 2016). PTSD is alleged to be associated with high rates of concurrent psychiatric disorders, particularly including, but not limited to, substance and alcohol/nicotine addictions and all kinds of depressive disorders (Williamson et al. 2017; Bollinger et al. 2000; Keane and Wolfe 1990). Further, traumatic events triple the risk of developing subsequent psychotic experiences later in life; the effect persist after adjustment for the possible presence of a mental disorder preceding the psychotic post-traumatic episode, which points to a direct and strong association between PTSD and psychosis (McGrath et al. 2017).

Aside from the socio-psychological or psychiatric consequences, PTSD may also encompass debilitating somatic disorders. In this context, comorbid metabolic and hormonal sequelae are notably underscored (Morris et al. 2012). PTSD increases two-fold the risk to develop insulin-resistant diabetes type 2, and also is conducive to the development of obesity, and other atherosclerosis-related pathological conditions (Roberts et al. 2015). Although molecular phenomena linking such comorbid conditions to PTSD remain mostly conjectural, interestingly the common denominator seems to be a proinflammatory propensity endowed by PTSD (von Känel et al. 2007). Since somatic complications of PTSD may come to light in a variably and unpredictably delayed time scale, patients with the pathologies above outlined ought to be assiduously scrutinized in the process of anamnesis taking for the past history of a traumatic imbroglio to identify the biopsychosocial disease links.

PTSD has complex and multiple symptoms and is a highly impairing condition that imposes a significant economic and social burden (Hawkins et al. 2015; Kessler 2000). When coping with serious illness, choosing the right therapy is of key importance. However, treating patients suffering from PTSD poses a significant challenge and therapy still remains within the arcana of medical art. The existing guidelines for pharmacotherapy concern so broad and divergent groups of drugs, for instance, selective serotonin reuptake inhibitor (SSRI) like fluoxetine and related compounds, monoamine oxidase inhibitors like phenelzine, tetracyclic antidepressants like mitrazepin, antipsychotics like risperidone, and the list goes on (Cipriani et al. 2017). Pharmacotherapy should be individually tailored, taking into account the background history and current disease manifestations, with the placebo effect being sometimes the best therapeutic solution.

2 Meditation Interventions in Post-traumatic Stress Disorder

Since the available evidence is not robust enough to suggest any pharmacotherapy of PTSD of finite efficacy, psychotherapeutic interventions have come to the fore as a prioritized option (Bisson and Andrew 2007; Schäfer and Najavits 2007). A variety of psychotherapy treatments have been developed for PTSD, such as trauma-focused cognitive behavioral therapy, stress management, or eye movement desensitization and reprocessing; the therapies that also include cognitive group treatment. Among the psychological interventions, meditation has been recognized as one of the notably effective modes. Meditation is an empirically-validated treatment for PTSD. A growing body of evidence suggests that meditation-based interventions have the potential to reduce symptoms and improve mood and general well-being (Mitchell et al. 2014; Seppälä et al. 2014). Further, meditation enhances openness to experience, one of the personality traits, which is associated with improvement in coping with stress by decreasing avoidance-oriented attitude to stressful situations and with better control of one's emotions (Pokorski and Suchorzynska 2018).

Meditation is a mind-to-body practice. It is an essential element in all of the world's major contemplative, spiritual, and philosophical traditions (Walsh 1999; Shapiro et al. 2008). According to Manocha (2000) meditation is a discrete and well-defined experience of a state of 'thoughtless awareness' or mental silence, in which the activity of the mind is minimized without reducing the level of alertness. Walsh and Shapiro (2006) described meditation as the self-regulation practices that aim to bring mental processes under voluntary control through focusing attention and awareness. The effects of meditation on health are based on the principle of mind-to-body connection and there is a growing body of literature showing the efficacy of meditation in various health related problems (Hussain and Bhushan 2010). Mind-to-body practices are increasingly used in the treatment of PTSD and are associated with a positive influence on the stress-induced illnesses such as depression and PTSD in most existing studies (Kim et al. 2013). As described by Cloitre et al. (2011) meditation has been identified as an effective second-line approach for emotional, attentional, and behavioral (e.g., aggression) disturbances in PTSD. Lang et al. (2012) further suggest the meditation as a therapeutic intervention for PTSD.

Anapanasati meditation, which is a concentrative meditation that focuses on one's respiration and suppresses other thoughts, is a tool for exploring subtle awareness of mind and life. Mindfulness of breathing helps oxygenate the body, reduces stress and anxiety, and induces peace of mind (Deo et al. 2015). The meditator is able to focus attention and see the impermanence of his experiences, which nullifies the feeling of being destroyed by them. Breathing interventions boost emotion regulatory processes in healthy populations (Arch and Craske 2006). Sack et al. (2004) have indicated that breathing-based meditation practices may be beneficial for PTSD. Seppälä et al. (2014) have reported that breathing-based meditation decreases posttraumatic stress disorder symptoms in US military veterans.

Mindfulness meditation, which is a sensitive non-concentrative type of meditation that notices things and picks up the object of attention as it pleases, helps reduce the level of stress in PTSD patients by cultivating awareness and acceptance of dysfunctional mental behaviors and helping change emotional experiences (Steinberg and Eisner 2015). The term 'mindfulness' has been used to refer to a psychological state of awareness, a practice that promotes this awareness, a mode of processing information, and a characterological trait. Germer et al. (2005) defines mindfulness as moment-by-moment awareness. The evidence concurs that mindfulness helps develop effective emotion regulation in the brain (Davis and Hayes 2011; Siegel 2007). Mindfulness is associated with low levels of neuroticism,

anxiety, and depressive symptoms, as well as high levels of self-esteem and satisfaction with life (Tanner et al. 2009; Brown and Ryan 2003). Mindfulness meditation is indicated in PTSD as it leads to positive outcomes in trauma survivors (Christelle et al. 2014; Follett et al. 2006).

Likewise, Vedananupassana meditation or awareness of sensations and feelings is a form of mindfulness meditation which is useful in the treatment of PTSD. Chronic pain and PTSD commonly co-occur in the aftermath of a traumatic event (Palyo and Beck 2005). In addition, both are mutually maintaining conditions, and pain sensations can trigger PTSD symptoms (Sharp and Harvey 2001). People with chronic pain and co-morbid PTSD report poorer quality of life (Morasco et al. 2013). Vedananupassana meditation is beneficial in alleviating pain in the individuals with PTSD.

Loving-kindness meditation is a practice designed to enhance feelings of kindness and compassion for self and others. Self-compassion is considered a promising change mode of behavioral approach in the treatment of PTSD (Hoffart et al. 2015). Kearney et al. (2014) have conducted a loving-kindness meditation study in 42 military veterans with active PTSD and found the effect of increased positive emotions. According to Kearney et al. (2013), this kind of meditation appears safe and acceptable, and is associated with reduced symptoms of PTSD and depression. Hinton et al. (2013) have demonstrated that loving-kindness meditation has a potential to increase emotional flexibility and to decrease rumination. It may regulate emotional stability and form a new adaptive processing mode centered on psychological flexibility.

Research has shown that transcendental meditation can also be an effective technique to treat PTSD. Transcendental meditation is derived from the ancient yoga teaching (Lansky and St. Louis 2006). It is a purely mental technique that falls within the category of 'automatic self-transcending' because the practice allows the mind to effortlessly settle inward, beyond thought, to experience the source of thought, pure awareness (Rees 2011; Travis and Shear 2010). Chhatre et al. (2013) have described transcendental meditation as a behavioral stress reduction program that incorporates mind-to-body approach, and demonstrated its effectiveness in improving outcomes through stress reduction. Rees et al. (2013) have shown a reduction in posttraumatic stress symptoms in Congolese refugees practicing transcendental meditation. Rosenthal et al. (2011) have highlighted the successful use of transcendental meditation on the veterans of Operation Enduring Freedom and Operation Iraqi Freedom suffering from PTSD. Further, Orme-Johnson and Barnes (2014) have explored a reduction in anxiety in response to transcendental meditation.

The therapeutic added value of meditation may be its hypnotic-like effect. Zazen, 'seated meditation' in which the body and mind are calmed, has an apparent hypnotic influence as evidenced by blocking the cortical alpha wave EGG response to repeated click stimuli (Kasamatsu and Tomio 1966). Hypnogenic engagement of attention with imaginary resources prevents the perception of the sense of reality and hinders the passage of external painful remembrances (Tellegen and Atkinson 1974), with understandably beneficial effects in PTSD. Hypnotherapy alone has a beneficial effect on PTSD symptoms. The largest to-date meta-analysis on the subject, performed on the selected 6 studies covering 391 subjects, has demonstrated positive effects of hypnotherapeutic techniques specifically related to avoidance and intrusion, and to overall PTSD symptomatology (O'Toole et al. 2016). Meditation-associated hypnosis, although seldom by far considered for PTSD treatment, appears to be of distinct efficacy (Lesmana et al. 2009).

3 Conclusions

PTSD is a psycho-physiologic-behavioral disorder, which calls for psychobehavioral ways of treatment. Meditation is an important part of health and spiritual practice. It is a form of mental exercise that has an extensive therapeutic value. There are three major types of meditative practices: mindfulness of breathing, non-concentrative mindfulness, and transcendental meditation. Due to a multitude of

meditative techniques and approaches, it is hard to average meditations together to define the underlying mechanisms and clinical benefits. The difficulty consists in the paucity of verifiable research, small sample sizes of patients, variable methods of meditation, setting different outcome measures, and other issues. Despite these shortcomings, empirical evidence accumulates to demonstrate that meditation is associated with overall reduction in PTSD symptoms, and it improves mental and somatic quality of life of PTSD patients. Meditation interventions seem justifiable as an adjunct to the ill-defined polypharmacy arsenal in PTSD treatment or a standalone trial in otherwise failed treatment efforts of this multimodal disease.

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Rehabilitation of Neuromotor Disabilities in Aquatic Microgravity Environment

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Abstract

The aquatic environment has a high potential in rehabilitation treatment of acute lesions and in chronic diseases. The Safe Bearing Back method is proposed to stimulate the reorganization of deteriorated sensory neuromotor skills. The aim of the present study was to verify the effectiveness and the long-term maintenance of the benefits of a specific thermal rehabilitation training in neuromotor and neurological disabilities. Seventy four patients were evaluated using the Functional Independence Measure (FIM), Tinetti Gait-Balance Scale (TIN), and Visual Analog Scale (VAS) for pain. In addition, a general health index was developed, conceived as a linear combination, with unit weights, of the normalized FIM, TIN, and VAS indicators. Measurements were made at T1 (baseline before treatment), T2 (after a five-month treatment, which was the end of treatment), and T3 (6 months after the end of treatment). Self-sufficiency, walking ability, and subjective pain perception were improved after the treatment. The improvement tapered off during the six-month-long follow-up, but the patients' condition remained well compared with the baseline level before the implementation of the treatment program. We conclude that hydrokinesitherapy with the Safe Bearing Back method demonstrates is clearly effective in the immediate and medium-term rehabilitation of neuromotor diseases.

Keywords

Aquatic microgravity environment · Chronic disease · Health index · Hydrokinesitherapy · Neuromotor disabilities · Rehabilitation · Treatment program

1 Introduction

Many studies have shown that the aquatic environment has a high potential for rehabilitation in the treatment of acute lesions and in chronic diseases, but nowadays its use is rather modest. Several studies have supported rehabilitation therapy in the aquatic microgravity environment to improve static and dynamic balance in a variety

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of pathological conditions (Marinho-Buzelli et al. 2017; Carere and Orr 2016; Barker et al. 2014; Hall et al. 2008). Water immersion can be considered a form of sensory and mechanical agitation applied to patients. The analgesic effect of water is due to heat and buoyancy that are able to block nociception by acting on mechanical and heat receptors, and thus also on the mechanisms of spinal segmental transmission (Lange et al. 2006; Bender et al. 2005). During immersion in hot water, body's temperature rises, causing a reduction in gamma fiber activity; consequently reducing muscle activity and spasticity. These effects increase a range of motion and improve the muscle-joint alignment, which is conducive to rehabilitation efficacy (Furnari et al. 2014; Bellomo et al. 2012).

In rehabilitation of neurological pathologies of both adult and pediatric age, the focus is on four main objectives: proprioceptive training, recruitment of synergic muscles, reduction of hyperreflexia, and spasticity, and muscle strengthening (Lambeck 2001; Gehlson et al. 1984). The benefits of hydrokinesitherapy have to do with a reduction in the level of load, which provides it a better working environment in case of postural instability or anomalous load distribution (Kesiktas et al. 2004). Spasticity is one of the main causes of disability and is usually a challenge in the management of various neurological disorders. During the development of spasticity, the spinal cord undergoes changes in motor neuron excitability, interneuronal connections, and reflexes (Stein 2004). That also involves a modification in muscle tone, which likely results from alterations in the ascending reticulo-spinal pathways and spinal cord interneuronal circuits, along with dysfunction of the corticospinal system. Consequently, segmental imbalance, alterations in inhibitory control, and neuronal sprouting are observed (Ballaz et al. 2011). Pharmacologically, spasticity is often cured with baclofen, a central agonist of $GABA_B$ receptors, belonging to the class of amino-butyric acid derivatives. Baclofen is the most commonly used drug and is usually administered orally or intrathecally as a liquid preparation administration (Rekand 2010). The predominant effect of $GABA_B$ receptor modulation is a reduction of calcium cellular inflow that inhibits the release of excitatory neurotransmitters, including glutamate and aspartate. Baclofen relaxes the muscles and, consequently, improves painful spasms and clones (Faraj 2017). The most commonly reported side effects in clinical trials, particularly in the elderly or patients with cognitive impairment are sedation, drowsiness, weakness, paresthesia, and nausea and vomiting (Brennan and Whittle 2008).

Thanks to therapies in a water microgravity environment, it is possible to reduce the intake of drugs, without being confronted with their side effects. The goal of some neurological rehabilitation techniques is to provide additional sensory information to the patient about bodily oscillations and spatial orientation (Yalcinkaya et al. 2014). Water immersion is considered the only sensory perturbation applied to those who stand in the water (Pöyhönen and Avela 2002). Visual sensory deprivation, while remaining in the aquatic environment, could potentially lead to further instability as shown in previous studies in a different scenario of sensory perturbation (Kjellgren and Westman 2014). Considering the immersion mechanisms in maintaining the postural stability, aquatic rehabilitation programs can be developed in a targeted manner (Louder et al. 2014). Microgravity, combined with specific peripheral receptor stimuli, plays a fundamental role in the technique used (Marinho-Buzelli et al. 2017). The maintenance of a standing position in neurological pathologies is a complex task that is achieved through the integration of sensory information from the visual, vestibular, and somatosensory systems (Bellomo et al. 2012). A way to manage neuromotor disability is to supplement or replace the limited, altered, or missing sensory information by providing additional information to the central nervous system *via* alternative stimulation, which aims at restoring balance (Jung et al. 2017). The aquatic microgravity environment provides is one way to provide such an alternative mechanical input for proprioceptive training to maintain posture. This kind of environment is indicated for treatment of Parkinson's disease, particularly in the initial stage where there is not yet major stiffness and the walking ability is still preserved (Ayán and Cancela 2012).

Buoyancy is one of the physical properties of water that provides postural support and reduces the load on the joints, which enables the patients with cerebral palsy to resume independent moving (Kesiktas et al. 2004). Buoyancy is a force that helps sustain body motion in the water, which underlines the efficacy of hydrotherapy in patients with different physical disabilities such as amputation, cerebral palsy, and paraplegia (Ballaz et al. 2011; Kelly and Darrah 2005). Hydrotherapy also improves respiratory and cardio-respiratory functions, self-esteem, and self-awareness (Prins 2009; Sheldahl et al. 1987). The literature shows that rehabilitation in a water microgravity environment is useful not only in postural rehabilitation, but also in neuromotor and neurocognitive treatment (Schaefer et al. 2016; Šrámek et al. 2000).

The goal of this experimental study was to demonstrate the immediate effects and their medium-term upholding of a sequential therapeutic approach that includes motor and manual therapy in the aquatic environment. To pursue this goal, we introduced an innovative general health index (GHI), which consists of a linear combination of the most common measures of the assessment of autonomy, balance, gait, and pain.

2 Methods

2.1 Study Design

The study was conducted in accordance with the Declaration of Helsinki for Human Research and a local Ethics Committee's indications. This clinical trial was conducted in the Department of Physical Medicine and Rehabilitation of the "G. d'Annunzio" University of Chieti-Pescara in collaboration with 'Fonte Della Salute La Cavallina' Thermal Rehabilitation Center of Castelnuovo della Daunia (FG) in Italy. The study sample consisted of 74 patients with neuromotor disabilities who were divided into four subgroups with the following pathological features:

- Group I: 18 patients with infant cerebral palsy (PCI), with mild mental retardation and limb spasticity;
- Group II: 18 chronic stroke patients, with spasticity but no mental retardation;
- Group III: 10 patients with peripheral nerve injury and 8 with inflammatory degenerative joint processes, with no spasticity or mental retardation, predominantly with orthopedic and rheumatic problems;
- Group IV: 20 patients with Down syndrome, with strong mental retardation.

The following inclusion criteria were applied:

- Neuromotor disability certified by the National Healthcare System of $\geq$67%; the Italian lower cut-off to attest the presence of disability;
- Score of Mini-Mental State Examination (MMSE) $\geq$18; a time-proven and internationally recognized test for the assessment of intellectual and cognitive deterioration. A total score is between 0 and 30 points. A score <18 indicates a serious cognitive impairment, $\geq$18 and $\leq$25 a moderate impairment, and $\geq$26 cognitive normality (Folstein et al. 1975).

Exclusion criteria were as follows: severe cardiopathies, infections and mycosis, hypersensitivity to chlorine, fever, open wounds, urinary incontinence, and serious cognitive disability.

2.2 Study Procedures and Outcome Measures

Measurements and questionnaire evaluation was performed at the following sampling times of the study protocol: T1 (baseline before treatment), T2 (after a five-month treatment, which was the end of treatment), and T3 (6 months after the end of treatment) outlined above. The following questionnaires were used as the outcome measures:

1. Functional Independence Measure (FIM) to assess the daily-life activities related to personal care, sphincter control, commuting mobility, locomotion, interpersonal

communication, cognitive and memory skills; ≤18 points = disability; 126 = self-sufficiency.
2. Tinetti (TIN) scale, also known as the Performance-Oriented Mobility Assessment (POMA) to assess the balance stability and cognition in healthy or moderate dementia patients. The scale is concerned with the position changes, balance maneuvers, and the movement aspects for safe and efficient running of daily-life activities; the scale consists of two sections: one for balance and one for gait; 0 = patient cannot walk alone; 28 = patient proficient in walking.
3. Visual Analog Scale (VAS) for the evaluation of painful perception, with 0 = absence of pain; 10 = maximum pain.

Moreover, D-VAS-2 and D-VAS-3, D-FIM-2 and D-FIM-3, and D-TIN-2 and D-TIN-3 indicate the differences between T2-T1 and T3-T2 time points of measurements.

2.3 Intervention Protocol

All patients were treated with the Safe Bearing Back method. This method is an integrated rehabilitation technique consisting of a peripheral sensorineural stimulation performed in an aquatic microgravity environment, using sequential motor education and myofascial manual therapy. The patients underwent five treatment cycles consisting of 20 daily sessions, lasting for 30 min each. The 30-min treatment consisted of the following stages:

- ≈ 5 min of adaptation to the aquatic environment and relaxation;
- ≈ 5 min dedicated to walking;
- ≈ 10 min dedicated to neuromuscular manual therapy, focusing attention on primary trigger points;
- ≈ 10 min dedicated to proper joint mobility, proprioceptive increase and muscle strengthening.

In this study, drawing on the literature (Baena-Beato et al. 2014) and also on our own experience, we used a protocol consisting of five weekly aquatic rehabilitation sessions, with the alternating phases of static and dynamic work phases and the adaptation of external workloads, according to the individual characteristics of each patient. Therapy in a microgravitary aquatic-thermal environment is a great alternative for all those patients who have a high risk of falling and report joint pain that limit the patient in walking (Arnold et al. 2008). The analgesic effects of hydrotherapy have also been studied in patients with arthralgia developing in the course of hormonal therapy; with an overall decrease in pain perception being documented (Baena-Beato et al. 2014; Cantarero-Villanueva et al. 2013). In addition, the efficacy of hydrotherapy is also reported concerning the balance, posture, and walking ability in elderly patients (Ayán and Cancela 2012; Momberg et al. 2008; Rissel 1987) and in patients with orthopedic pathologies. There is also improvement in pain relief in prosthetic patients and in patients with neurological problems (Valtonen et al. 2010; Zamarioli et al. 2008). The Safe Bearing Back rehabilitation method used in the present study is an algo-osteo-myofascial approach that involves specific receptor-mediated motor and tissue stimulation, activation of neural reflexes, and the use of biomechanical load principles for soft tissue, all in the aquatic microgravity environment (Bellomo et al. 2012; Saggini et al. 2008).

3 Statistical Evaluation

Data were collected at the T1, T2, and T3 time points to determine how the patients, treated during the hydrotherapy period, recovered and maintained the cognitive and motor skills afterwards. Differences in the mean values of FIM, TIN, and VAS scores at these time points, for each questionnaire, were evaluated with one-way ANOVA for repeated measurements, followed by Tukey's *post hoc* test. Normality of data distribution was tested with the Jarque-Bera test while the covariance sphericity with the

Mauchly test. The FIM and TIN data violated the normality and sphericity assumptions, so that these data were subjected to the following transformation to meet the normality and sphericity assumptions:

$$\mathrm{FIM}_{\mathrm{trasf}} = \mathrm{Log}\left[\left(\max_{(\mathbf{FIM})} + 1\right) - (\mathrm{FIM})\right]$$
$$\mathrm{TIN}_{\mathrm{trasf}} = \mathrm{Log}\left[\left(\max_{(\mathbf{TIN})} + 1\right) - (\mathrm{TIN})\right]$$

To jointly consider the aspects of FIM, TIN and VAS variables, a general health index (GHI) was introduced. This index is designed as a linear combination, with unitary weights, as follows:

$$\mathrm{GHI} = \alpha \bullet \mathrm{FIM} + \beta \bullet \mathrm{TIN} + \gamma \bullet \mathrm{VAS}$$

where $0 \leq \mathrm{GHI} \leq 3$, and $0 \leq \alpha, \beta, \gamma \leq 1$ (in this case, we consider $\alpha, \beta, \gamma = 1$). FIM, TIN, and VAS scores were normalized, yielding.

$$0 \leq \mathrm{FIM}, \mathrm{TIN}, \mathrm{VAS} \leq 1$$

For the GHI, VAS score was inverted to ensure its accordance with the FIM and TIN variables (i.e., higher VAS values after the inversion should be interpreted as an improvement in the subjective perception of pain; the VAS values now being in line with FIM and TIN values that directly corresponded to patients' improvement). Of note, VAS values were not present for patients belonging to Group IV, because they were unable to give a reliable answer to the subjective perception of pain due to severe mental retardation. Hence, GHI was calculated for a 54 unit sample. The GHI is a measure that varies between a minimum of 0 and maximum of 3 because it consists of three sub-indicators, each ranging from 0 to 1. Thus, GHI equalled zero when a patient had serious shortcomings in self-sufficiency and walking, and had a strong perception of pain. The index equalled three the patient was not suffering from severe pain and has a high degree of independence and the ability to walk. Differences in GHI were evaluated with one-way ANOVA and the *post hoc* test as above outlined. A p-value <0.05 defined statistically significant differences. The tests were performed with the R statistical software.

4 Results

Table 1 shows the descriptive analysis of variables at T1, T2 and T3 time points of measurements. The minimum patient age is 12 years and the maximum is 83 years. The sample is composed by 47% of males and of the mean age of is 50.4 years ±19.3. The mean of FIM score was 87.9 at T1, with a minimum of 18 and a maximum of 124 points. The mean TIN score was 14.3 ± 7.1 at T1, with a minimum of 0 and a maximum of 25 points. The mean VAS score was 8.3 ± 1.4 at T1, with a minimum of 5 and maximum of 10 points. This last score was calculated in a smaller sample of 54 patients, since Group IV patients were excluded due to severe cognitive disability, making it impossible to obtain subjective responses.

Table 2 contains the correlation coefficients between each variable and the others for combinations of ten variables. The variables correspond to patient age and the three questionnaires' data, each obtained at the three time points of measurements. The arrangement of

Table 1 Questionnaire scores – continuous data

Observation	n	Mean ± SD	Median (Min–Max)
Age (year)	74	50.4 ± 19.3	54 (12–83)
FIM-1	74	87.9 ± 28.3	96.5 (18–124)
FIM-2	74	100.1 ± 27.7	110 (21–126)
FIM-3	74	93.45 ± 28.8	104 (19–126)
TIN-1	74	14.3 ± 7.1	16 (0–25)
TIN-2	74	17.8 ± 8.1	20 (0–28)
TIN-3	74	16.4 ± 7.8	18.5 (0–28)
VAS-1	54	8.3 ± 1.4	8.5 (5–10)
VAS-2	54	4.7 ± 1.6	5 (1–9)
VAS-3	54	6.5 ± 1.7	7 (2–10)
D-FIM-2		12.2 ± 12.1	8.5 (2–84)
D-FIM-3		−6.7 ± 10.9	−4 (−80–0)
D-TIN-2		3.5 ± 2.5	3 (0–11)
D-TIN-2		−1.4 ± 2.4	−1 (−10–10)
D-VAS-2		−2.7 ± 1.9	−3 (−6–0)
D-VAS-3		1.4 ± 1.3	1 (−1–25)

FIM-1, FIM-2, and FIM-3; TIN-1, TIN-2, and TIN-3; VAS-1, VAS-3, and VAS-3 are Functional Independence Measure scale, Tinetti scale, and Visual Analog Scale, respectively, at T1, T2, and T3 time measurement points. D-FIM-2 and D-FIM-3, D-TIN-2 and D-TIN-3, D-VAS-2 and D-VAS-3 indicate the differences between T2-T1 and T3-T2 time points of measurements

Table 2 Correlation matrices of variables – Bravais-Pearson's index with a list-wise deletion

	AGE	FIM-1	FIM-2	FIM-3	TIN-1	TIN-2	TIN-3	VAS-1	VAS-2	VAS-3
AGE		0.452*	0.468*	0.487*	0.437*	0.459*	0.442*	0.067	−0.009	0.098
FIM-1			0.964*	0.977*	0.799*	0.827*	0.812*	−0.002	0.112	0.067
FIM-2				0.986*	0.822*	0.853*	0.835*	0.031	0.134	0.105
FIM-3					0.830*	0.855*	0.844*	0.031	0.114	0.084
TIN-1						0.959*	0.970*	−0.001	0.067	0.018
TIN-2							0.974*	−0.027	0.010	−0.007
TIN-3								−0.001	0.040	0.015
VAS-1									0.747*	0.736*
VAS-2										0.777*
VAS-3										

FIM-1, FIM-2, and FIM-3; TIN-1, TIN-2, and TIN-3; VAS-1, VAS-3, and VAS-3 are Functional Independence Measure scale, Tinetti scale, and Visual Analog Scale, respectively, at T1, T2, and T3 time measurement points; *$p < 0.001$

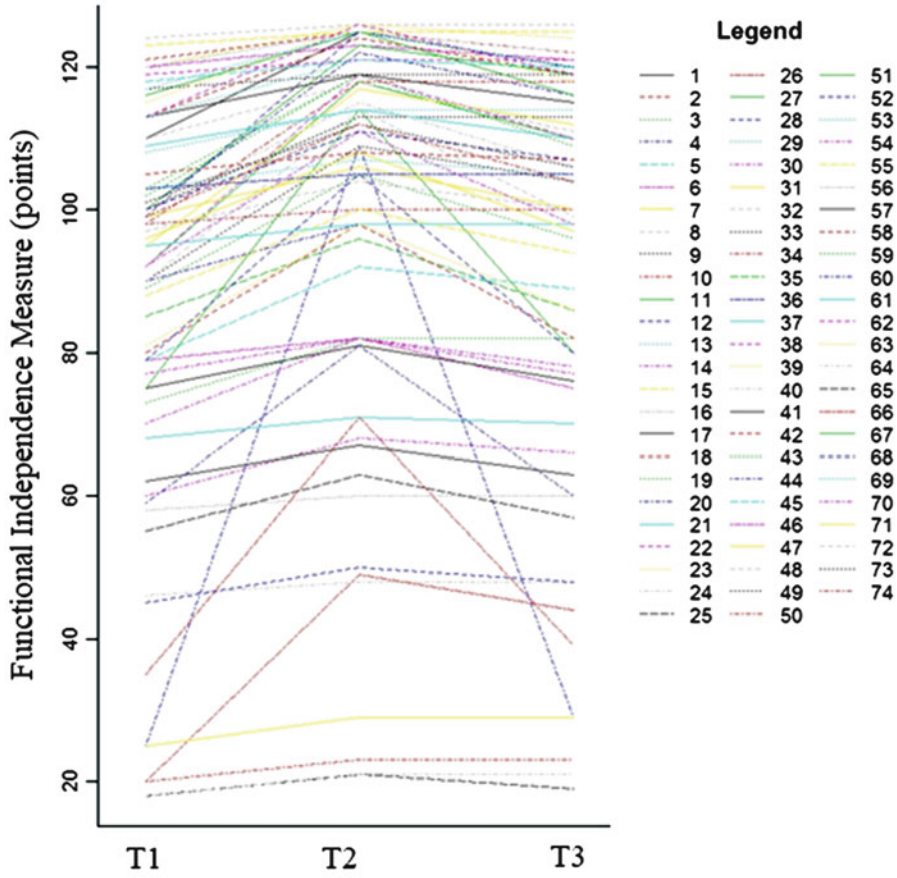

Fig. 1 Evolution of Functional Independence Measure (FIM) over treatment periods; T1 – baseline before treatment; T2 – after 5 months' treatment, i.e., end of treatment; and T3–6 months after the end of treatment

the table shows the correlation matrix examining the inter-dependence between pairs of variables. FIM and TIN scores showed strong positive correlations with AGE, but it was not the same for VAS. Figs. 1, 2, and 3 show the evolution over time of FIM, TIN, and VAS, respectively. These figures as well as Table 1 show that at T3 (6 months after end of treatment) patients got worse. In fact, FIM and TIN scores have a pyramid trend (Figs. 1 and 2), while VAS trend is the opposite (Fig. 2) in most patients.

Consecutive panels of Table 3 show the results of statistical analysis of ANOVA for repeated measures to assess differences among the data of FIM, TIN, VAS, and GHI scales, each obtained at the three time points of measurements. The analysis shows that time has a significant effect in each case. Therefore, the null hypothesis on the equality among the mean data for T1, T2, and T3 was rejected. The *post-hoc* tests show that the mean values of the three time variables were statistically different from each other in case of each scale.

Figure 4 presents the distribution of GHI index at T1 (red), T2 (green), and T3 (blue) time points. The representation shows that the index distribution at T2 is translated horizontally, compared to T1 and T3. This result is confirmed in Table 3 (bottom panel), which illustrates the results of ANOVA for repeated measures concerning the overall health index obtained at these three different time points. The plot confirms a general health improvement at the end of treatment period (T2) and a little decrease in health 6 months after treatment completion (T3).

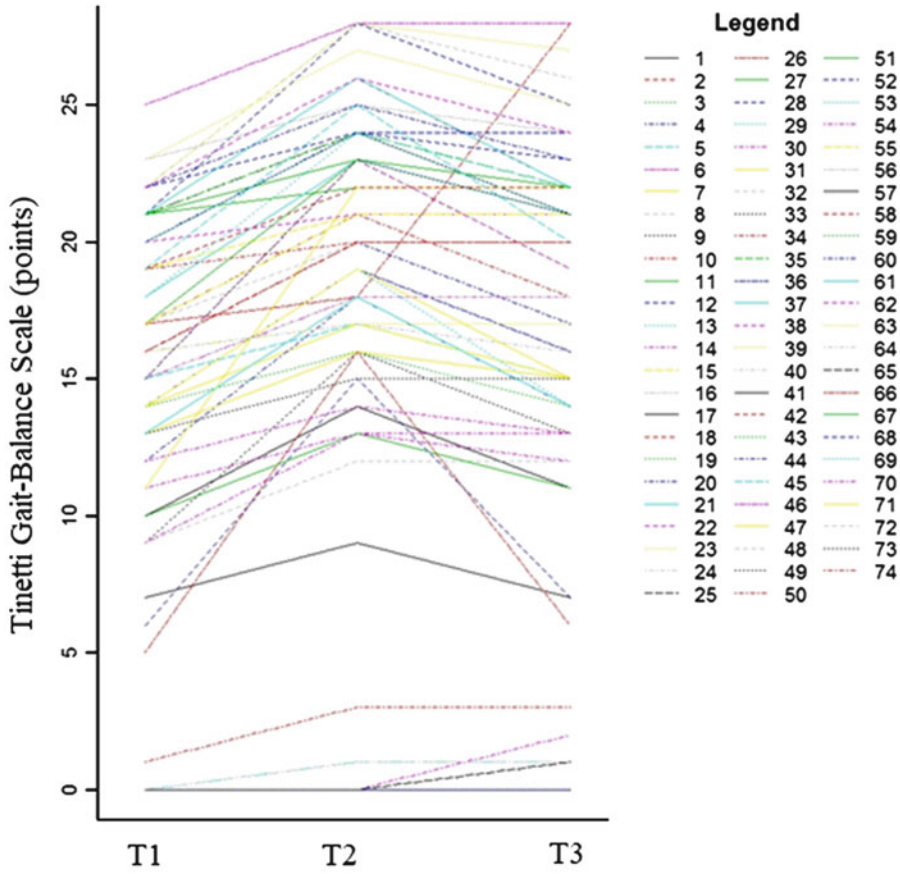

Fig. 2 Evolution of Tinetti Gait-Balance Scale (TIN) over treatment periods; T1 – baseline before treatment; T2 – after 5 months' treatment, i.e., end of treatment; and T3–6 months after the end of treatment

5 Discussion

Immersion in water produces a central redistribution of the blood volume, which decreases neural system activation (Friedman Elliot and Irwin 1997). A decrease in sympathetic activity, accompanied by increased parasympathetic activity, increases blood flow, accelerates cellular metabolism, removes fatty substances, and decreases pain sensitivity by promoting vasodilation and blood circulation (Forestier and Françon 2008). The autonomic nervous system remodeling positively affects the perception pain and fatigue associated with musculoskeletal disorders due mostly to decreasing muscle tension (Yasui et al. 2010). Several studies have highlighted exercise protocols that are associated with improved mobility and walking in neuromotor dysfunction (Pérez de la Cruz 2017; Kim et al. 2016; Lai et al. 2015; Motl et al. 2005). Individuals with neuromotor disabilities should be encouraged to undergo adjuvant therapy to mitigate progressive dysfunction of mobility, especially taking into account the prevailing physical inactivity among such patients.

The Safe Bearing Back aquatic program offers advantages in the treatment of neural and musculoskeletal diseases that cannot be achieved in hydrotherapy consisting of passive immersion only or balneotherapy (Saggini et al. 2008). In the present study, descriptive statistics shows that the scores of all three scales used improved at the end of treatment (T2); the FIM averaged about a 100, the TIN about 18, and the VAS about 5 points. These results show that the degree of self-sufficiency (FIM), walking ability (TIN), and

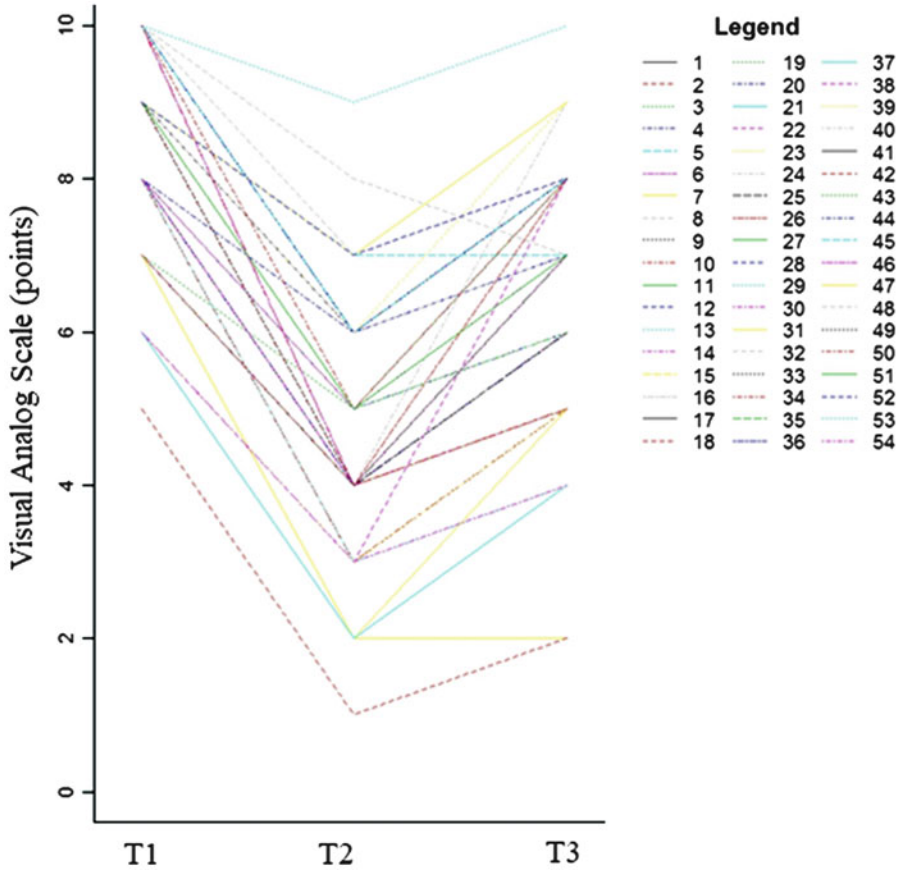

Fig. 3 Evolution of Visual Analog Scale (VAS) over treatment periods; T1 – baseline before treatment; T2 – after 5 months' treatment, i.e., end of treatment; and T3 – 6 months after the end of treatment

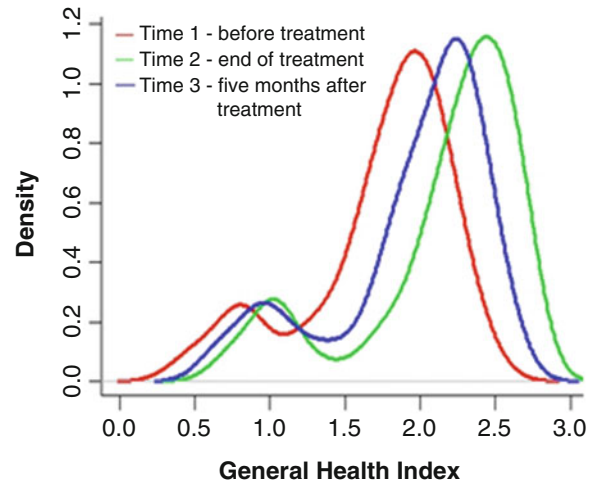

Fig. 4 Evolution of general health index (GHI) distribution over time

Table 3 ANOVA analysis for repeated measures (within) for differences among the scores in FIM, TIN, VAS, and GHI scales, each obtained at three time points of measurements

FIM – Functional Independence Measure					
	DF	SS	MS	F-value	Pr > F
Time	2	18.900	9.449	67.84	< 2e-16*
Residuals	146	20.340	0.139		
Simultaneous tests for general linear hypotheses multiple comparisons of means: Tukey contrasts					
	Estimate	SE	z-value	Pr > |z|	
T2-T1	−0.712	0.061	−11.579	< 1e-06*	
T3-T1	−0.288	0.061	−4.688	6.88e-06*	
T3-T2	0.4228	0.061	6.891	< 1e-06*	
TIN – Tinetti gait-balance scale					
	DF	SS	MS	F-value	Pr > F
Time	2	7.244	3.622	41.62	< 4.97e-15*
Residuals	146	12.205	0.087		
Simultaneous tests for general linear hypotheses multiple comparisons of means: Tukey contrasts					
	Estimate	SE	z-value	Pr > |z|	
T2-T1	−0.441	0.049	−9.096	< 1e-04*	
T3-T1	−0.250	0.049	−5.164	< 1e-04*	
T3-T2	0.191	0.049	3.932	0.000265*	
VAS – visual analog scale					
	DF	SS	MS	F-value	Pr > F
Time	2	363.0	181.5	290.1	< 2e-16*
Residuals	106	66.3	0.63		
Simultaneous tests for general linear hypotheses multiple comparisons of means: Tukey contrasts					
	Estimate	SE	z-value	Pr > |z|	
T2-T1	−3.667	0.152	−24.09	< 2e-16*	
T3-T1	−1.815	0.152	−11.92	< 2e-16*	
T3-T2	1.852	0.152	12.16	< 2e-16*	
GHI – General health index					
	DF	SS	MS	F-value	Pr > F
Time	2	4.529	2.264	299.5	< 2e-16*
Residuals	106	0.801	0.008		
Simultaneous tests for general linear hypotheses multiple comparisons of means: Tukey contrasts					
	Estimate	SE	z-value	Pr > |z|	
T2-T1	0.409	0.167	24.47	< 2e-16*	
T3-T1	0.212	0.167	12.68	< 2e-16*	
T3-T2	−0.197	0.167	−11.79	< 2e-16*	

DF, degrees of freedom; *SS,* sum of squares; *MS*, mean square; *Pr* > *F*, the significance probability value associated with the F-value; SE, standard error; *Pr* > |*z*|, the significance probability value associated with the z-value. T1 (baseline before treatment), T2 (after a five-month treatment, i.e., end of treatment), and T3 (6 months after end of treatment); *p < 0.001

subjective pain perception (VAS) all were significantly improved after water microgravity-related treatment (from T1 to T2). In addition, the study underscores that the condition of patients started to decline after stopping of treatment (from T2 to T3), although it still remained at a significantly better level than the baseline one (from T1 and T3). Therefore, considering a moderately long period of 6 months after hydrotherapy cessation, we can state that the neurosomatic and neurophysiological stimulation, resulting from the Safe Bearing Back protocol in the aquatic microgravity environment, is of benefits to patients' autonomy, gait-balance, and the psychoemotional sphere of pain perception, which is also tantamount to improvement in general health status.

The improvement in mobility achieved by the Safe Bearing Back aquatic program and manual therapy can be exploited by clinicians and physiotherapists to promote new strategies for correcting physical agility in dysfunctional patients (Motl et al. 2005). Aquatic exercise therapy improves mobility in all types of neuromotor disabilities, particularly in progressive and resistant to pharmacotherapy cases (Rekand 2010). The present findings are in line with those of Baena-Beato et al. (2014) who have demonstrated that a program of intense, five sessions a week, rehabilitation in a water microgravity environment is well tolerated and produces significant improvements in both pain perception and quality of life in patients with chronic pain (Baena-Beato et al. 2014). Moreover, LeFort and Hannah (1994) have highlighted that aerobic exercise in the aquatic environment is beneficial for both cardiovascular and articular mobility, and for psychological aspect as it reduces anxiety and enhances mood that is constantly low in chronic, particularly neurodegenerative, conditions. Giaquinto et. al. (2007) have reported that gait in the physically fit elderly assumes characteristics comparable to those in younger persons during hydrotherapy. Likewise, Alcade et al. (2017) have reported beneficial effects of aquatic rehabilitation exercises in patients with knee osteorthritis. The strength of the present study was to demonstrate the effectiveness of a Safe Bearing Back Sequential Motor Method with respect to specific outcomes such as stroke, balance of autonomy, and pain in patients with neuromotor disabilities. The weakness of the study is represented by a non-complete homogeneity of the sample group concerning individual pathologies and different age of patients belonging to the same group. Nonetheless, we believe that the findings lend support for those other studies that point to the applicability of a specific training in the aquatic microgravity in neuromotor disabilities.

6 Conclusions

Aquatic exercise can refer to pool therapy, hydrotherapy, or balneotherapy. Hydrotherapy is frequently applied to patients with painful neurological or musculoskeletal alterations, because the heat and floatability of water can block nociceptors by acting on thermal receptors and mechanoreceptors and exert a positive effect on spinal segmental mechanisms (Bellomo et al. 2012; Geytenbeek 2002). Warm water can also increase the blood flow, helping to dissipate allogeneic chemicals and to enhance muscle relaxation. Finally, the hydrostatic effect of water can alleviate pain by reducing peripheral edema and sympathetic nervous system activity. A systematic review on crenobalneotherapy in patients with limb osteoarthritis found that it reduces pain and improves function and quality of life (Forestier and Françon 2008). These water features allow the physiotherapist to work differently from treatments outside the water and to propose exercises that would not be possible in a terrestrial environment, such as articular techniques associated with tissue manipulation in water (Oh et al. 2015). The main aim of the present article was to determine the effectiveness of hydrotherapy to modify pain, quality of life, and other symptoms in neuromotor disease (Marinho-Buzelli et al. 2017; Konrad et al. 1992). The findings demonstrate appreciable improvements after treatment in both the Tinetti Gait-Balance Scale and the Visual Analog Scale, which were maintained, albeit gradually abating, during the six-month-long follow-up period. Even though there was a significant reduction of the beneficial effects at the end of the follow-up, corrective changes in the gait, balance, autonomy, and pain remained, to an extent, compared with the baseline level present before the commencement of treatment. Therefore, given the clearly advantageous effects of hydrokinesitherapy with the Safe Bearing Back method employed in this study, we suggest that five therapeutic cycles for each patient, could be administered not in 5 months, but distributed over the solar year. That might ensure a longer term maintenance of improvement in the health condition of patients with neuromotor and neurological disabilities, and in effect lower both social and economic costs of patient care. Regarding the General Health Index, our future research will focus on the use of new functional tools to analyze the

positive trend over time (Maturo and Di Battista 2018) and the significance of changes in the advanced methodological context (Di Battista et al. 2016).

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

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Lifestyle Intervention Program for Amelioration of Dysmetabolism in Overweight Women

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Abstract

Overweight and obesity, a cluster of multiple risk factors for atherosclerosis such as elevated blood pressure, elevated glucose level, and dyslipidemia, increase the risk of all-cause mortality and cardiovascular morbidity and mortality. Physical activity and a proper diet are essential preventive measures. The aim of the study was to evaluate the effects of a two-month intervention program consisting of a low-caloric diet (1,500 kcal) and increased physical activity on the anthropometric parameters, body composition, resting metabolic rate, and maximum oxygen uptake. The study was conducted in 22 women aged 20–38 with diagnosed overweight or obesity. We found that after completing the eight-week-long intervention program, there were significant changes in body composition, consisting of a smaller proportion of body fat and increased lean body mass. Further, we observed a decrease in body weight by 4.3 ± 2.5 kg ($p < 0.01$), a reduction in waist and hip circumference of 2.6 ± 4.5 cm ($p < 0.01$) and 4.4 ± 2.9 cm ($p < 0.01$), respectively, and an increase in maximum oxygen uptake by about 5.2 ± 8.4 ml/kg/min ($p < 0.01$). We conclude that the intervention program consisting of counseling on diet and physical activity may be highly motivational for patients with excess body weight and care givers should give it a try before commencing more aggressive psychopharmacological therapies.

Keywords

Diet · Dysmetabolism · Body composition · Obesity · Overweight · Physical activity

1 Introduction

Obesity is defined as a state of excess adiposity that presents a risk to health, such as increased risk of chronic diseases, including cardiovascular disorders, type 2 diabetes, or cancer. It is a consequence of sustained positive energy intake over time (Romieu et al. 2017; Anderson et al. 2015). Excessive weight gain is caused by many factors, such as genetic endowment, and environmental,

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endocrine, behavioral, and psychological factors. High energy density of food, sweet drinks, and low physical activity are notably suggested as being obesogenic (Fresan et al. 2016). Although the causes of overweight and obesity may vary, the basis for a therapeutic intervention is a reduction of body weight through lifestyle changes, including a low-energy diet and increased physical activity (Sweeting and Caterson 2017). The difference between energy intake and expenditure, frequently referred to as energy balance, has become of great interest because of its direct relation to a long-term gain or loss of adipose tissue. A reduced proportion of body fat and increased lean body mass are conducive to physical fitness and, more importantly, to quality of life (Bhutani et al. 2017). The introduction of healthy eating habits based on the supply of all necessary ingredients in the right proportions, regular meals, increased consumption of whole grain products, fruits and vegetables, adequate fluid intake and regular physical activity are the most effective way to obtain desired results (Fogelholm et al. 2017; Hever and Cronise 2017). There is an apparent need for interventions which can improve access to healthy diets and healthy food choices. There has been an increasing global recognition of the necessity of effective strategies to prevent and control overweight and obesity.

2 Methods

The study was conducted in a targeted group of 31 women, 22 of whom completed the study. The mean age of study participants was 24 ± 4 years, range of 20–40 years, body mass index (BMI) of 28.0 ± 3.7 kg/m^2, and height of 166 ± 7 cm. The inclusion criteria were as follows: body mass index (BMI) ≥ 25 m/kg^2, age, lack of chronic diseases, and the willingness to undertake regular physical activity and a diet therapy. Taking dietary supplements and smoking tobacco excluded participation in the study. The research involved the use of a low-energy diet (1500 kcal/day) prepared by the authors and a regular physical activity for 8 weeks. The participants were taught by a dietitian how to prepare meals following the rules of a low-energy diet in which the total energy was provided from 20% protein, 30% fat, and 50% carbohydrates. Attention was also drawn to adequate supply of calcium – 1000 mg (Donnelly et al. 2013; WHO Guidelines 2010). The recommended physical activity consisted of minimum 30 min of exercise daily for 5 days a week, with a moderate intensity (heart rate of 60–80% of the maximum heart rate). The exercise included a weight-lifting training at least twice a week. The following parameters were the subject of assessment before and after the eight-week long intervention: diet, physical activity, body composition, maximum oxygen uptake (VO_2max), anthropometric data, body weight, and waist and hips circumferences.

Body composition was examined in the supine position, using a bioelectrical impedance (BIA) method, with a Bodystat 1500 MDD device (Ballakaap; Isle of Man, British Isles). The evaluation was performed before a meal, at least 2 h after fluid intake, not less than 12 h after active exercise or 24 h after drinking alcohol, coffee or caffeinated beverages (Mulasi et al. 2015). Resting metabolic rate was measured in kcal/day using a Fitmate Med cardiopulmonary setup (Cosmed; Rome, Italy) and oxygen uptake at rest was measured using a specialized flow meter for free movement. Exercise capacity was assessed at an ergometer, with maximum oxygen uptake expressed in ml/kg/min in a submaximal test (test protocol 20 W/min).

Data were expressed as means $\pm$ SD. The results obtained after the eight-week-long study protocol, were compared with the baseline levels, using a *t*-test for dependent variables. Associations between variables were evaluated with Pearson's correlation coefficient. A p-value ≤ 0.05 defined statistically significant differences. The evaluation was conducted with a Statistica v10 commercial package (StatSoft; Tulsa, OK).

3 Results

3.1 Consumption of Energy, Macronutrients, and Physical Activity

The study participants reduced daily energy intake to 1,506 ± 353 kcal; a mean decrease by 354 ± 399 kcal ($p < 0.002$). The percentage of energy delivered from carbohydrates in the diet before and after the intervention remained at a comparable level; a change by −1.1 ± 5.8%, ($p < 0.42$). The percentage of energy delivered from fat decreased by 5.4 ± 4.6% ($p < 0.001$) and that from protein increased by 4.3 ± 3.4% ($p < 0.001$). The amount of each macronutrient in the diet, both before and during the dietary intervention, is presented in Table 1. The consumption of calcium remained stable. The participants' physical activity amounted, on average, to 307 ± 121 min *per* week (Fig. 1).

Physical activity performed during the eight-week-long intervention program made the maximum oxygen uptake increase by 5.2 ± 8.4 ml/kg/min ($p < 0.01$) (Fig. 2).

3.2 Anthropometric Measurements

Body weight before onset of study was 77.4 ± 10 kg and it decreased by 4.3 ± 2.5 kg ($p < 0.01$) after the eight-week-long physical program; the mean decrease amounted to 5.5 ± 3.2%. BMI decreased from 28.0 ± 3.7 kg/m^2 to 26.3 ± 3.5 kg/m^2 ($p < 0.01$). Dietary and physical activity interventions reduced the waist and hip circumferences by 4.5 ± 2.6 cm and 4.4 ± 2.9 cm, respectively ($p < 0.01$ both). Waist circumference decreased from 110.5 ± 8.3 cm before to 106 ± 6.8 cm after the interventions ($p < 0.01$).

Table 1 Daily food composition before and during the lifestyle intervention program

	Before intervention	During intervention	p-value
Protein (g)	72.0 ± 17.4	73.5 ± 16.5	0.710
Fat (g)	67.1 ± 19.8	45.2 ± 17.8	0.001
Carbohydrates (g)	248.9 ± 54.0	212.3 ± 50.1	0.070
Calcium (mg)	783.0 ± 176.6	871.1 ± 331.9	0.320

Data are means ±SD

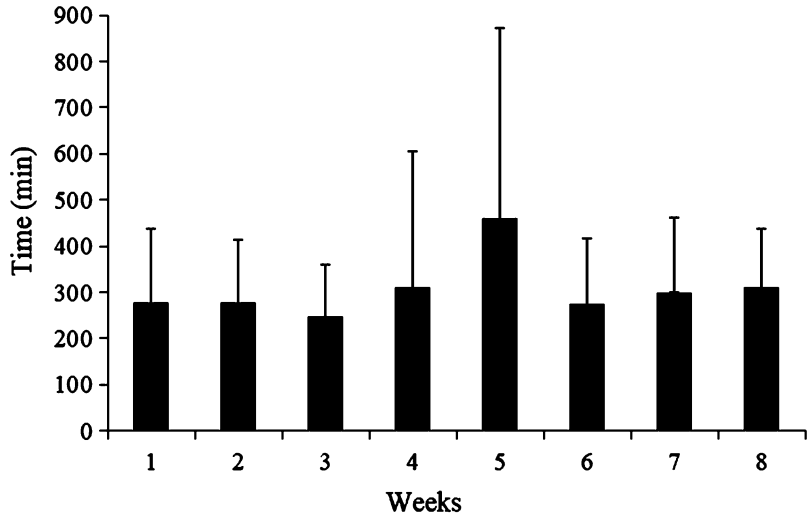

Fig. 1 Physical activity of study participants during the eight-week-long lifestyle intervention program. Data are means ±SD

There were changes in body composition after completing the physical program. The proportion of body fat decreased from 33.9 ± 5.3% to 31.7 ± 5.6%, which is equivalent to the loss of 3.0 ± 2.0 kg of fat mass ($p < 0.01$). As a result, the percentage of lean body mass increased by 2.2 ± 1.8% ($p < 0.01$). Further, a proportion of body water in the women participating in the program increased by 1.4 ± 1.4% ($p < 0.01$). The resting metabolic rate remained unchanged, amounting to 1,632 ± 232 kcal/day before and 1,576 ± 248 kcal/day after completion of the intervention program ($p = 0.33$).

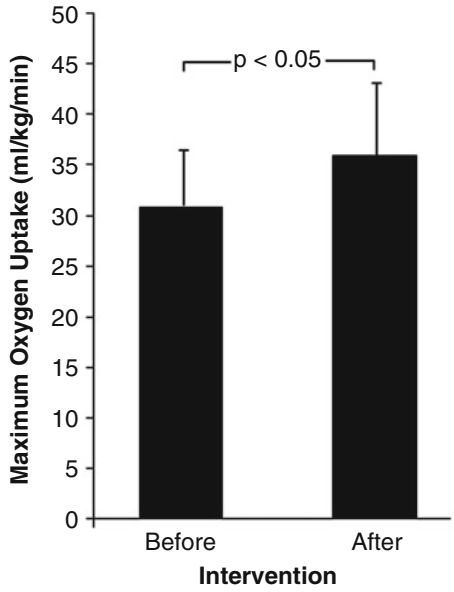

Fig. 2 Influence of physical activity on maximum oxygen uptake (n = 20)

The energy intake correlated with weight loss in women, assessed at the end of the diet and physical activity intervention program ($r = 0.57$, $p < 0.05$) (Fig. 3). Hip circumference at the end of the intervention program correlated inversely with body weight present before the intervention program; i.e., the higher was the body mass at the beginning, the greater the loss in hip circumference ($r = 0.51$; $p < 0.05$) (Fig. 4). Finally, body fat mass correlated with changes in body mass (Fig. 5).

4 Discussion

A working assumption in the present study was that energy intake should come from the following sources: 50% from carbohydrates, 30% from fat, and 20% from protein. The results demonstrate that carbohydrates covered about 54% of energy intake, both before and during the physical intervention program. Interestingly, the

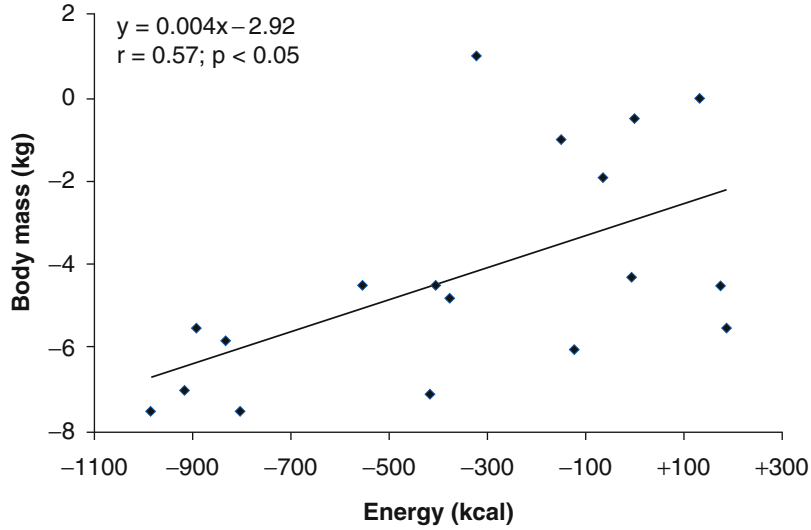

Fig. 3 Relationship between energy intake and weight loss in women at the end of the intervention program

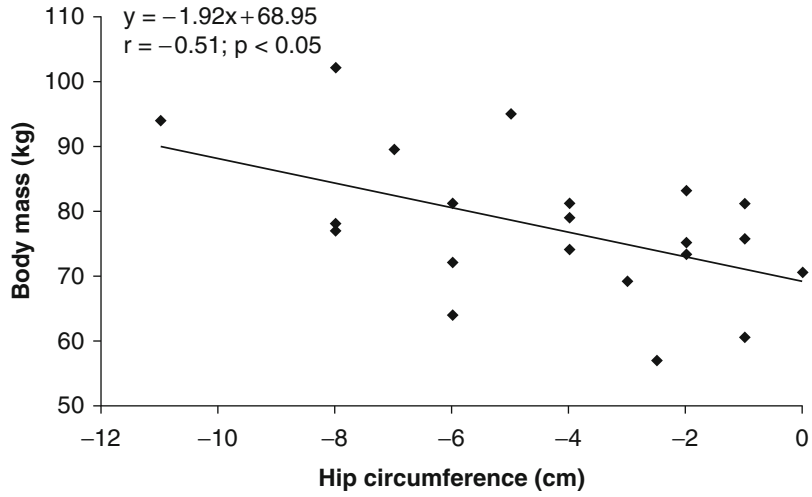

Fig. 4 Relationship between hip circumference at the end of the intervention program and body mass onset of program

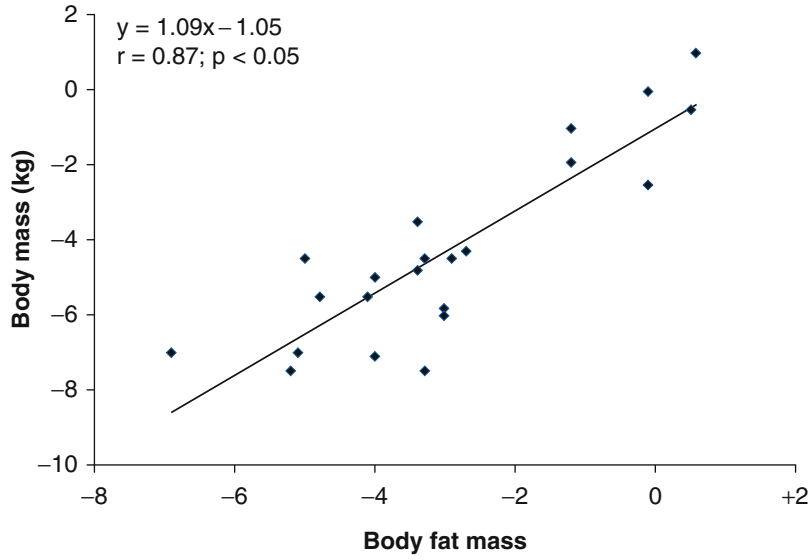

Fig. 5 Relationship between body fat and weight loss in women

quality of carbohydrates changed as the consumption of complex carbohydrates increased. Protein consumption in our patients made up slightly more than 20% of energy intake. Protein intake was not tightly controlled as some studies suggest that this intake may be conducive to weight loss, reduction of appetite, and consequently also lean body mass (Jager et al. 2017; Kim et al. 2016). Layman et al. (2005) have showed that increasing dietary protein intake to 30% of the total energy intake in active women results in fat loss by more than 2%, compared with a diet containing 18% of protein. The greatest changes observed in the present study were, however, in the consumption of fat. Fat intake decreased, on average, by was 22 ± 17 g. Since 1 g of fat provides nine kilocalories, this decrease lowered energy content of the diet by about 200 kcal. A reduction in fat intake was identified as one of the main factors facilitating weight reduction in a group of more than 4,000 Americans in 2012 (Raatz et al. 2017).

Calcium consumption plays an important role in weight loss and reduction in waist circumference (Lee and Cho 2017; Tremblay and Gilbert 2011; Torres et al. 2010). It also reduces blood pressure and helps reduce body fat. Although we conducted educational instruction in the women of the present study concerning the intake of food rich in calcium, it failed to bring expected results. A daily intake of calcium increased by about 100 mg during the diet intervention, which failed to reach statistical significance and remained below the widely recommended daily intake of 1,000 mg calcium.

The dietary and physical intervention used reduced body mass by 4.3 ± 2.5 kg during the eight-week-long study, which corresponds to 0.54 kg *per* week and corresponds to the weight reduction recommendations of 0.5–1 kg *per* week (Garvey et al. 2016). A comparable weight loss of 4.3 ± 3.4 kg was obtained during the same time period in a group of overweight women in a study of Volek et al. (2002). In the present study, percentage of weight loss constituted 5–10% of body weight, with a mean of 5.4%. A similar 5.9% loss of body weight was observed by Kerksick et al. (2010) in a ten-week-long period with the intervention based on a diet of 1,200 kcal *per* day and physical activity undertaken three times a week. However, Connolly et al. (1999) have conducted a study in 31 overweight women, which lasted for 12 weeks and was based on the 1,194 kcal diet consisting of 70% of carbohydrates, 15% of protein, and 15% of fat, and a variable combination of physical activity. Those authors observed a 6.2 kg body weight reduction in women who were on the diet alone, a 6.8 kg reduction in those who were on both diet and aerobics performed three times a week, and a 7.0 kg reduction in those who were on the diet combined with aerobics and weight training. However, comparative studies are difficult since weight reduction depends on the diet, duration and type of physical activity, duration of intervention, and energy restrictions.

In this study, women's waist and hip circumference was reduced by 4.5 cm and 4.4 cm, respectively. Kerksick et al. (2010) have reported a 5.7 reduction in waist circumference in a study with more restrictive diet intervention (−300 kcal), lasting for 2 weeks longer. In turn, Ross et al. (2004) have reported a somehow greater reduction in waist circumference of 6.5 cm after a 14-week-long intervention in women whose physical activity expenditure amounted to 500 kcal daily. Other authors have also suggested that the best results in waist circumference reduction are observed in patients who are on a diet combined with enhanced physical activity (Mastellos et al. 2014; Washburn et al. 2014). An average reduction of fat mass, we herein report, amounted to 3.0 kg or 2.1% of body weight. This result seems satisfying in comparison with a study by Volek et al. (2002) who have reported a fat mass reduction of 2.5 kg or 1% of the body weight. Likewise, a longer, three-week lasting diet intervention in obese sedentary women a study of Kerksick et al. (2010) led to the 3.1 kg or 1.6% of body weight reduction of fat mass. We found a decrease of 55.7 ± 25.2 kcal/day in resting metabolic rate in the overweight women during the diet and physical intervention program; the decrease was at the level of an insignificant trend. Other studies show an association between the resting metabolic rate and lean body mass (Stiegler and Cunliffe 2006). Christensen et al. (2017) have shown that resting metabolic rate does not change in response to different types of training in women with chronic diseases over a 13-month-long intervention, while Schubert et al. (2017) have shown a 4% increase in the rate in subjects treated with sprint interval training during 4 weeks.

To enhance physical fitness, it is necessary to introduce regular physical activity. In our study, VO_2max increased from 30.8 ± 5.5 ml/kg/min before to 36.0 ± 7.1 ml/kg/min after the physical intervention program ($p < 0.05$), on average, by 5.2 ± 8.4 ml/kg/min or 17% within 8 weeks of the physical training. This effect was achieved through the introduction of about 5 h of moderate physical activity a week, which follows the international guidelines to maintain health benefits (WHO 2010).

We conclude that the intervention program introduced to overweight women, consisting of low-caloric diet and increased physical activity,

resulted in a smaller proportion of body fat and increased lean body mass, a loss of body weight, a reduction in waist and hip circumference, and an increase in maximum oxygen uptake, along with increased physical fitness. Such positive results may be motivational for patients and should be tried out before commencing more aggressive psychopharmacological therapies.

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

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Immunization Coverage in Children with Inflammatory Bowel Disease

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Abstract

Patients suffering from inflammatory bowel diseases (IBD) are at increased risk of infections, mainly due to immunosuppressive treatment. Moreover, infections may cause flares of IBD. Vaccination is the most effective way of preventing many infections. The aim of this study was to evaluate the vaccination status of Polish children with IBD. Individual immunization cards of children with IBD and healthy controls were reviewed. Demographic data such as age, sex, and IBD history, including therapy type, were collected. We enrolled 267 children into the study, including 214 children with IBD and 53 controls. None of the children had completed the full up-to-date routine childhood immunization schedule recommended in Poland. Controls were more than 4 times more likely to be vaccinated than the IBD patients, with the vaccines that enjoy the insurance reimbursed (OR 4.1, 95% CI 2.2–7.9). In conclusion, the study demonstrates a poor vaccination status in children suffering from IBD.

Keywords

Crohn's disease · Immunization · Immunosuppressive treatment · Inflammatory bowel disease · Ulcerative colitis · Vaccination

1 Introduction

Inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn's disease (CD), are multifactorial diseases with genetic heterogeneity. It is hypothesized that IBD is a result of impaired or altered immune response to environmental and infectious triggers (Weersma et al. 2007). Long-term treatment of IBD is based on the use of anti-inflammatory agents, corticosteroids, immunomodulators, such as thiopurines or methotrexate, and on biological therapy. Due to immunosuppressive treatment, children with IBD are considered to be immunocompromised. The course of IBD in children is usually more severe and complicated than that in adults. Therefore, the majority of children with IBD are treated more aggressively with a combination of immunosuppresive and immunomodulatory agents.

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The available literature data suggest that IBD patients are at higher risk of infections, also the infections that could be prevented with vaccination. The increase risk of infection is related, apart from immunosuppressive therapy, to the underlying disease, malnutrition, and surgical procedures (Davies 2016; Aberra and Lichtenstein 2005). It is also known that any infection may trigger a flare of IBD. Therefore, protecting this group of patients against infections is of upmost importance. The most effective way to protect against infectious diseases is immunization. In line with this strategy, a committee formed by the Crohn's and Colitis Foundation of America in 2004 and the European Crohn's and Colitis Organization formed in 2014 have stated that IBD patients would benefit from immunization for vaccine-preventable diseases. The committees recommend the use of inactivated vaccines for all patients with IBD, regardless the type of therapy (Rahier et al. 2014; Sands et al. 2004). Live vaccines are recommended only for those patients who are not treated with immunosuppressive agents.

Until now, only a few studies assessing vaccination status have been conducted in children and adolescents with IBD (Fleurier et al. 2015; Longuet et al. 2014; Soon et al. 2013; Crawford et al. 2011). None of those studies had a control group. Further, none were performed in Eastern Europe. This applies also to Poland, where many vaccines are strongly recommended but are not reimbursed, even for immunocompromised children such as those with IBD. In addition, in East European countries, social care is usually limited, and the majority of everyday costs related to chronic diseases such as transportation, special diets, dietary supplements, or rehabilitation, are covered by parents. Taking these facts together, we can hypothesize that vaccination coverage among children suffering from IBD may be inadequate. Thus, the aim of the present study was to assess the immunization status in pediatric IBD patients in Poland.

2 Methods

This study was conducted in two university-affiliated pediatric hospitals in Warsaw and Cracow, Poland, between September 2015 and September 2016. Children with IBD were diagnosed according to the revised Porto criteria, which include the assessment of clinical symptoms and the results of laboratory, radiological, endoscopic, and histologic findings (Levine et al. 2014). The type of IBD treatment was recorded. Patients treated with immunomodulators (azathioprine 2.5–3 mg/kg/day, or 6-mercaptopurine 1.5 mg/kg/day, or methotrexate 1.5 mg/m^2/week for 12 weeks or more) and anti-tumor necrosis factor-α biological therapy (any regimen including infliximab or adalimumab) were considered to be immunosuppressed. Combination therapy was defined as a concomitant use of immunomodulators and biologics. Healthy children, recruited among patients admitted to the hospital due to sporadic acute infection but without any chronic disorders, made up the control group.

Individual immunization cards of all included patients were reviewed. The immunization record was defined as complete when it included all routine childhood immunizations, and both the vaccine type and number of shots were age-appropriate at the time of study onset. As an example, if a child received 2 doses of hepatitis B vaccine instead of 3 recommended doses, we classified such vaccination as incomplete. In Poland, reimbursed vaccination scheme includes 3 doses of hepatitis B vaccine, 1 dose of tuberculosis vaccine, 7 doses of tetanus and diphtheria vaccine, 6 doses of pertussis vaccine, 4 doses of *Haemophilus influenzae* B vaccine, 4 doses of polio vaccine, and 2 doses of measles, mumps, and rubella vaccine. Demographic data such as sex, age, and IBD history, including therapy type, were collected.

The Shapiro-Wilk test of data distribution was applied. The Mann-Whitney U test was used to compare continuous variables. Proportions were

evaluated by χ^2 test. A log-linear analysis was used to assess the relationship between more than two categorical variables. A p-value <0.05 defined statistically significant differences. Data were analyzed using a commercial statistical package of Statistica v13 (Statsoft; Tulsa, OK).

3 Results

We enrolled 267 children into the study; 214 children with IBD and 53 controls. Table 1 shows the baseline characteristics of the study groups and vaccination coverage by type of vaccine. The fully reimbursed vaccination schedule was received by 38/214 (17.8%) of children with IBD and 25/53 (47.2%) of controls. The likelihood of being vaccinated with all reimbursed vaccines was more than 4 times higher in controls than in children with IBD (OR 4.1, 95% CI 2.2–7.9). Patients with ulcerative colitis had a likelihood of completing the entire reimbursed vaccination schedule more than 2 times greater than controls (OR 2.6, 95%CI 1.3–5.3). There were some differences in therapy type between patients with ulcerative colitis and Crohn's disease as shown in Table 2. However, these differences failed to influence the completion of reimbursed vaccinations (p = 0.15).

None of the children received the full up-to-date routine childhood immunization schedule recommended in Poland. However, almost all children completed the full course of vaccination against hepatitis B (211/214 of IBD patients and 53/53 of controls). In the first 2 years of life, complete vaccination with combined vaccine against diphtheria, tetanus, whole-cell pertussis and the vaccine against polio were received by 93% and 95% of the children with IBD, respectively. At age of 6 years, these vaccines were received by 71% and 69%, respectively. Concerning the polio, there were 45% attenuated polio vaccine and 24% inactivated polio vaccine. At age 10 years, 85% of children received two

Table 1 Baseline characteristics of study groups and vaccination coverage by type of vaccine

	All IBD (CD+ UC) (*n = 214*)	Controls (*n = 53*)	p	Subtype of IBD		
				CD (*n = 131*)	UC (*n = 83*)	p
Median age (quartiles) (year)	14.5 (11.5–16.0)	11.0 (4.5–14)	**0.0001**	15.0 (12.5–16.0)	13.0 (10.0–16.0)	**0.020**
Sex (males)	117	29	0.90	81	36	**0.008**
Median disease duration (year)	2.0 (1–4)	–	–	2.0 (1–4)	1.5 (1–4)	0.60
Reimbursed vaccination; n (%)						
Hepatitis B	211 (99.5)	53 (100)	0.50	128 (99.2)	83 (100)	0.80
Diphteria/tetanus	191 (89.7)	44 (83.0)	0.30	119 (91.5%)	72 (86.8)	0.30
Pertussis	194 (91)	44 (83.0)	0.20	122 (93.1)	72 (86.8)	0.10
Polio	198 (92.5)	47 (88.7)	0.50	123 (93.9)	75 (90.4)	0.30
HIB	56 (26.2)	29 (54.7)	**0.0001**	24 (18.3)	32 (38.6)	**0.001**
MMR	152 (71.0)	40 (75.5)	0.50	89 (67.9)	63 (75.9)	0.20
Completed reimbursed vaccination	38 (17.8)	25 (47.2)	**0.0001**	16 (12.0)	22 (26.0)	**0.008**
Unreimbursed vaccination; n (%)						
Hepatitis A	34 (15.9)	5 (9.4)	0.20	20 (15.3)	14 (16.9)	0.80
Varicella zoster	5 (2.5)	9 (17.3)	**0.0001**	3 (2.4)	2 (2.5)	0.70
Pneumococcal	86 (40.2)	20 (37.7)	0.50	53 (40.4)	33 (39.7)	0.90
Meningococcal	26 (12.2)	10 (19.2)	0.20	18 (13.7)	8 (9.6)	0.40
Tick-borne meningitis	7 (3.3)	4 (7.7)	0.30	2 (1.5)	5 (6.0)	0.20

IBD inflammatory bowel diseases, *CD* Crohn's disease, *UC* ulcerative colitis, *HIB–Haemophilus influenzae* type b vaccine, *MMR* measles, mumps, and rubella

Table 2 Therapy types of patients with IBD

Therapy type	CD	UC	p
5-ASA	10 (7.8%)	29 (35.4%)	**0.0001**
Immunomodulators	66 (51.2%)	49 (59.8%)	0.20
Biologicals	3 (2.3%)	1 (1.2%)	1.00
Combo therapy	50 (38.8%)	3 (3.7%)	**0.0001**

CD Crohn's disease, *UC* ulcerative colitis, *5-ASA* aminosalicylate drugs

doses of the vaccine against measles, mumps, and rubella. At age of 14 years, 61% of children were vaccinated against diphtheria and tetanus, only 16% received inactivated polio vaccine, 18% – two doses of hepatitis A vaccine, 34% – 7-valent or 13-valent pneumococcal conjugated vaccine, 16% – 23-valent pneumococcal polysaccharide vaccine, and 14% – meningococcal C vaccine. Only 5% of girls were immunized against human papilloma virus at the recommended age.

4 Discussion

The results of this prospective study indicate that vaccination coverage is highly insufficient in the Polish pediatric IBD patients and much lower than in healthy controls. No child received a complete, up-to-date routine childhood immunization schedule as recommended in Poland. This finding is in line with the results of studies published in other countries, showing that a small percentage of pediatric patients who suffer from IBD keep abreast with the nationally recommended vaccine schedules. Only did 18% (9/50) of Canadian children receive the recommended vaccinations at the time of IBD diagnosis (Nicol et al. 2017). A similar 24% vaccination rate was reported in a French study (Longuet et al. 2014). In contrast, however, a 90% immunization rate in children with inflammatory bowel diseases was reported in Alberta, Canada (Soon et al. 2013). The high rate might presumably result from a comprehensive publicly funded immunization program in Alberta. That seems a credible explanation in view of the fact that the more expensive vaccines, such as that against HIB or anti-pneumococcal, are most often omitted from the immunization schedule, which we also found in this study. Conversely, we found the highest compliance with anti-HBV vaccination. Thus, we strongly believe that the most important factor resulting in the extremely low immunization rate found is the lack of reimbursement of vaccine costs, which also holds for children with chronic inflammatory diseases, including IBD.

The immunization system has it that vaccines are separated into 'obligatory', which are reimbursed, and 'recommended', which are not. Consequently, parents may think that the latter are voluntary and thus less important or of secondary importance. Another possible reason is a lack of awareness that vaccines are even more important for patients with chronic diseases than for healthy individuals. Finally, low vaccination rate may be related to insufficient awareness of gastroenterologists regarding the immune status of IBD children, as shown by several studies (Yeung et al. 2012; Wasan et al. 2011), as well as poor communication between gastroenterologists and primary care physicians who are responsible for immunization. The recommendation to postpone immunization when a patient experience a flare of IBD often becomes tantamount to the immunization abandonment.

In this study, the majority of children with IBD (152 out of the 214) received two doses of MMR vaccine, which are administered at 13 months and then 10 years of age in Poland. The results of other studies assessing the MMR coverage are more optimistic than ours. MMR vaccine administration varies according to different national immunization schedules. For example, two studies carried out in France show that 78–91% of IBD patients receive two doses of MMR (Fleurier et al. 2015; Longuet et al. 2014). In France, the second dose of MMR is administered in the second year of life. According to Nicol et al. (2017), 86% of Canadian children with IBD are fully vaccinated against MMR. In Canada, the second dose of MMR is administered between the fourth and sixth year of age. Our patients, who were diagnosed with IBD before or around 10 years of age and started immunosuppressive therapy

shortly afterward, did not receive the second dose of MMR. As it is a live vaccine, those children may never receive the missing second dose, according to immunization guidelines for IBD patients. A possible solution could be a readjustment of the Polish immunization schedule so that the second MMR dose would be given at 2–4 or 4–6 years instead of 10 years of age. Such an approach could also be justified by a low prevalence of IBD in children younger than five–year–old.

Only were nine out of the 214 patients with IBD in the present study vaccinated against all recommended, unreimbursed vaccines. One of such vaccines is the pneumococcal vaccine. Forty percent of children were vaccinated against pneumococcal infection. However, there was no difference in the pneumococcal vaccination rate between children with IBD and control subjects. These results are close to those of the French studies where the rate of pneumococcal vaccination among children with IBD was 32% (Longuet et al. 2014) or 36% (Fleurier et al. 2015). In the present study, the likelihood of completing the entire reimbursed vaccination schedule was more than two times higher among patients with ulcerative colitis than in those with Crohn's disease. Differences in therapeutic regimens used for Crohn's disease and ulcerative colitis had an insignificant effect on the completion of reimbursed vaccination schedule. We found, however, that older age at diagnosis of children with Crohn's disease, compared with ulcerative colitis, made them better acquainted with the reimbursed vaccination schedule; the result being in line with the observations of Fleurier et al. (2015). Our findings point to a need of an immunization awareness campaign in Poland addressed to both physicians caring for patients with IBD and to patients and their families. Such campaigns have been quite successful in some other countries. In France, letters are sent out to general practitioners and families to remind about vaccination recommendations and to encourage the verification of vaccination status of children with IBD (Fleurier et al. 2015). A US study has demonstrated increased adherence to vaccination guidelines resulting from an educational intervention delivered by IBD nurse to adult patients, using an informational brochure and a vaccination card (Coenen et al. 2017).

The main shortcoming of our study is a relatively small sample of patients. Also, data on varicella zoster vaccination are only approximate, as we failed to perform the anti-varicella antibody evaluation. In conclusion, we demonstrate poor vaccination status in the majority of immunocompromised children with inflammatory bowel disease, which is no different between the patients with Crohn's disease and ulcerative colitis. There is an unmet need to implement educational vaccination strategies addressed to both physicians and patients' families.

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

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