Advances in Experimental Medicine and Biology 788

Mieczyslaw Pokorski *Editor*

Neurobiology of Respiration

Springer

Advances in Experimental Medicine and Biology

Volume 788

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Neurobiology of Respiration

Springer

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ISSN 0065-2598
ISBN 978-94-007-6626-6 ISBN 978-94-007-6627-3 (eBook)
DOI 10.1007/978-94-007-6627-3
Springer Dordrecht Heidelberg New York London

Library of Congress Control Number: 2013942978

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

The scientific study of the respiratory system has increased enormously during the last decades, principally due to advances in cellular and molecular biology. The progress has allowed respiratory researchers and clinicians to investigate, in much detail, the complex processes underlying the development, function or malfunction, and regulation of respiration. Respiration emerges as the product of the assemblage of neuronal activity, lung ventilation, and respiratory muscle-pump function. The drive to inspire comes ultimately from the oscillating activity of the brain stem neuronal network. This drive, however, is subject to constant incoming modifications from central and peripheral sensory inputs. At the core of these inputs lies molecular signaling, which thus is essential in the pathophysiology of human disease and therapeutic interventions. The intricacies of how molecular signals are translated into respiratory regulation in health and disease have only begun to be unfolded.

Neurobiology of Respiration contains updated material regarding the broad aspects of respiratory regulation. The book explores the mechanisms underlying the pathologies of the respiratory system. Children's respiratory ailments are richly represented as this group is especially vulnerable. The notable topics of interest include molecular aspects related to neuroactive substances, chemokines and proteins released by cells in various pathological conditions, infections exemplified by influenza-like illnesses, inflammatory conditions such as asthma or chronic obstructive pulmonary disease (COPD), respiratory allergy and occupational diseases, immunology, cardiovascular-respiratory disorders, and respiratory oncology. Another group of chapters have to do with the psychosomatic aspects of smoking and with the consequences of smoking for muscle catabolism, underlain by the activation of cytotoxic proteins. Yet, other chapters deal with the cognitive and neural fatigue, which astonishingly may be detected by changes in the exhaled breath content, and with restriction of respiratory muscle work and contractility, particularly when the muscles are under hyperventilatory strain. The book content also includes observational and interventional epidemiological studies. Particularly, the latter form the basis of translational medicine which is considered as an extension of evidence-based medicine. Translational research encompasses the basic and social sciences with the view of optimizing the efficiency of patient care and prophylactic measures to improve the health and quality of life. I trust that the coordinated topics outlined above will allow the readers ample exposure to the latest developments in both scientific and clinical sides of respiratory neurobiology.

The book is a blend of basic and clinical research, and it is thought to promote the translation of science into clinical practice. It combines chapters describing the areas of current research and clinical interest. The chapters present the original findings supported by carefully planned and executed controlled experiments. The book helps the reader keep up with the state-of-the-art knowledge about the active sub-disciplines of respiratory neurobiology and may thus become the comprehensive base of reference in the field of respiration for a long time to come and an enduring source of future research ideas. The progress in respiratory neurobiology is the only way to increase the understanding of the mechanisms of respiratory disorders and therefore to be able to use the evidence-based treatment, which is the desired end of research. The book is a required text for those interested to learn about current respiratory research. It also is an essential text for clinicians searching for 'bench to bedside' treatments of lung diseases.

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Contents

Altered Histone Deacetylase Activity and iNOS Expression in Cells Isolated from Induced Sputum of COPD Patients Treated with Tiotropium

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A. Holownia, R.M. Mroz, P. Wielgat, T. Skopinski, A. Kolodziejczyk, A. Sitko, E. Chyczewska, and J.J. Braszko

Abstract

Chronic obstructive pulmonary disease (COPD) is the only major disease with increasing death rate. In COPD, progressive reduction in quality of life is closely related to the increasing limitation of airflow due to chronic bronchitis, cell hyperplasia, fibrosis, and irreversible lung damage. Signaling pathways involved in inflammatory processes in COPD and inflammatory response to therapy are unknown. Our aim was to isolate cells from induced sputum of COPD patients treated with formoterol or formoterol + tiotropium and assess enzymatic activity of histone deacetylases (HDACs) acetylated histone 4 (AcH4) and expression of inducible nitric oxide synthase (iNOS). HDACs are important in signal transduction and inflammation. iNOS is generating nitric oxide (NO) relevant to blood pressure regulation, inflammation and infections. Thirty stable COPD patients (21 males and 9 females, mean age 67 years) receiving 12 μg b.i.d. formoterol were assayed before and after 3 months add-on therapy consisting of 18 μg q.i.d. tiotropium. In all patients, spirometry, lung volumes, and DLCO were performed before and after tiotropium therapy and all patients were subjected to sputum induction. Sputum cells were isolated and processed to obtain cytosolic and nuclear fractions. HDAC activity was measured in nuclear fraction using colorimetric assay. Expression AcH4 and iNOS was quantified using Western blot. In patients receiving both drugs, FEV1 and lung volumes significantly improved compared with formoterol–only treated patients. Mean HDAC activity was slightly decreased ($P < 0.05$), while AcH4 levels and

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_1,

iNOS expression were significantly elevated in tiotropium-treated patients (increase by about 65 %; $P < 0.01$ and 77 %; $P < 0.01$ respectively). Our data show that beneficial effects of tiotropium in add-on therapy to formoterol may be related to altered histone signaling and increased iNOS expression.

Keywords
COPD • HDAC • iNOS • Induced sputum • Tiotropium

1 Introduction

Chronic obstructive pulmonary disease (COPD) is the only major disease with increasing death rate and an important health problem. In COPD, progressive reduction in quality of life is closely related to the increasing limitation of airflow due to chronic bronchitis, cell hyperplasia, fibrosis, narrowing of the airways, and irreversible lung damage. Apart from inflammation, additional airflow obstruction in COPD is caused by increased activity of parasympathetic system (Viegi et al. 2007). In pharmacotherapy of the disease several drugs are used, particularly long acting anticholinergics, like tiotropium or beta-2 mimetics like formoterol (GOLD 2008; Kaur et al. 2008). At early stages of the disease, a single inhaled tiotropium dose usually reverses compromised respiratory function due to relatively high affinity and low internal activity of the drug to muscarinic M3 and M1 receptors regulating both mucus secretion and vagally-induced contraction of airway smooth muscles (Kaur et al. 2008; Kato et al. 2006). Inflammation in COPD may also be related to increased acetylcholine levels. Published data from clinical and experimental studies (Santus et al. 2012) and our earlier results (Holownia et al. 2010, 2013) indicate that tiotropium increases antiinflammatory signaling and possibly also decreases airway inflammation and remodeling. In the current approach, our aim was to asses in cells isolated from induced sputum of COPD patients, before and after add-on tiotropium therapy, two important elements of nuclear and cytosolic inflammatory signaling – enzymatic activity of histone deacetylases (HDAC) and the level of acetylated histone H4 as well as cytosolic expression of inducible nitric oxide synthase (iNOS). HDACs are important in COPD because altered activity of HDAC enzymes affects chromatin remodeling and alters inflammatory gene transcription regulated by histone acetylation/deacetylation (Yao and Rahman 2012). iNOS produces nitric oxide (NO) involved in blood pressure regulation and in functional regulation of respiratory system (Malerba and Montuschi 2012). NO is usually increased in inflammation (Malerba and Montuschi 2012). iNOS is expressed both in lung epithelial cells, inflammatory cells, and also in skeletal muscle and experimental data suggest that it may serve as a therapeutic target in COPD (Marques et al. 2012).

2 Methods

2.1 Subjects and Treatment

Thirty patients included into the study gave their consent after a full discussion of the nature of the study, which had been approved by a Local Ethics Committee. All patients were stable COPD patients (21 males and 9 females, mean age 67 years) and were characterized with respect to sex, age, smoking history, COPD symptoms, comorbidity, and current medical treatment. Patients had stable disease, defined according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines including airflow limitation (FEV1 < 80 % predicted, FEV1/FVC < 70 %, GOLD stage 2–3). No patient in the study had symptoms nor

was treated for COPD exacerbation during at least 2 months preceding the day of inclusion. Exclusion criteria included other systemic diseases, other lung diseases apart from COPD, and lung tumors, pulmonary infection and antibiotic treatment 4 week before inclusion or inhaled/oral glucocorticosteroids in the 3 months before inclusion. Spirometry and lung volumes were performed with body box (Elite DL, Medgraphics, USA). The measurement was performed using standard protocols according to the American Thoracic Society guidelines.

All patients underwent 4 weeks' washout therapy with Salbutamol. After that, they were treated for 4 weeks with 12 μg b.i.d. formoterol and then subjected to the sputum induction. Subsequently all patients were treated for 3 months with add-on 18 μg q.i.d. Tiotropium and their sputum was collected again.

2.2 Sputum Induction and Processing

Sputum was induced by the inhalation of a 4.5 % hypertonic aerosol saline solution, generated by an ultrasonic nebulizer (Voyager, Secura Nova; Warsaw, Poland). Three flow volume curves were performed before and after each inhalation, and the best FEV1 was recorded. Induction of sputum was stopped if the FEV1 value fell by at least 20 % from baseline or if troublesome symptoms occurred. Samples were processed within about 15 min after termination of the induction. Samples were solubilized in equal volumes of 0.1 % dithiothreitol (Sigma Chemicals, Poznan, Poland) in Hanks solution, and incubated for 15 min in an ice bath. Cell suspension was then rinsed twice with Hanks solution, filtered by a nylon membrane and centrifuged (1,000 rpm) on Histopaque 1,077. Isolated cells were homogenized in a lysis buffer containing 10 mM N-2-hydroxyethylpiperazine-N′-ethane sulfonic acid, 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM ethylenediamine-tetraacetic acid, 0.2 mM NaF, 50 mM β-glycerophosphate, a protease inhibitor tablet, 0.2 mM Na-orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotenin and 10 % Nonidet P-40. Thereafter, the samples were incubated on ice for 15 min and then centrifuged at 13,000 × *g* for 30 sec. The cell pellets containing nuclei were retained and resuspended in extracting buffer (50 mM N-2-hydroxyethyl piperazine-N′-ethane sulfonic acid, 50 mM KCl, 300 mM NaCl, 10 % glycerol, 1 mM dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid, 0.2 mM NaF, 0.2 mM orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotenin, 50 mM β-glycerophosphate and a protease inhibitor tablet (Complete Mini; Roche Diagnostics, Mannheim, Germany)). The samples were then incubated on a rotating platform for 30 min at 4 °C followed by centrifugation at 13,000 × *g* for 5 min. The resulting nuclear extract and cytosol were evaluated for the activity of HDAC and iNOS expression, respectively. To assess AcH4 histone extraction was performed in cells treated for 30 min in ice with lysis buffer 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 1.5 mM phenylmethylsulfonyl fluoride and hydrochloric acid at a final concentration of 0.2 M and subsequently, lysed cells were centrifuged at 11,000 × g for 10 min at 4 °C. Supernatant containing acid-soluble proteins was dialyzed for 1 h, against 0.1 M acetic acid and then overnight against H_2O and frozen until assayed (Chadee et al. 1999).

iNOS and AcH4 were analyzed by sodium duodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)/immunoblotting with specific monoclonal antibodies recognizing human iNOS protein (Abcam, UK) or acetylated histone H4 (Upstate Biotech., UK). iNOS was run on 10 % SDS gels while AcH4 proteins were separated in 20 % polyacrylamide gels along with molecular weight markers (Bio-Rad, Hercules, CA) and loading controls. Gels were transferred onto 0.45 μm PVDF membranes (BioRad, Warsaw, Poland). Species-specific alkaline phosphatase secondary antibodies were purchased from Sigma (Sigma, Poznan, Poland). Protein bands were quantified using Quantity One software (BioRad, Warsaw, Poland).

HDAC activity was measured in nuclear fraction using colorimetric assay (Enzo Life Sci.

HDAC kit, Switzerland) adapted to cell homogenate (Yang et al. 2006). Protein levels were measured using a BCA kit (Sigma-Aldrich, Poznan, Poland).

Statistical analysis was performed using a statistical package – Statistica (Statsoft, Cracow, Poland) using nonparametric Wilcoxon test for paired data. The data were expressed as means ± SD. $P < 0.05$ was considered as statistically significant.

3 Results

Table 1.1 and Fig. 1.1 show respiratory parameters: forced expiratory volume in 1 s (FEV1) and inspiratory capacity (IC) in COPD patients before and after 3 months of tiotropium add-on therapy. Therapy improved clinical status of COPD patients and ameliorated their individual respiratory parameters. After 3 months of tiotropium, both FEV1 and CV increased by about 11 % ($P < 0.05$) compared with the corresponding values before tiotropium therapy.

Table 1.1 and Fig. 1.2 show biochemical data: specific nuclear HDAC activity and AcH4 levels as well as cytosolic iNOS expression in cells isolated from induced sputum of COPD patients before and after add-on tiotropium therapy. After tiotropium specific activity of HDAC was slightly lower ($P < 0.05$), while AcH4 levels were significantly elevated (by about 65 %; $P < 0.01$). Expression of iNOS was also increased (by about 77 %; $P < 0.01$).

4 Discussion

In COPD, an irreversible and progressive disease, reduced lung function is associated with local and systemic inflammation (Viegi et al. 2007; GOLD 2008). COPD patients are usually treated with bronchodilators such as long-acting beta-2 agonists frequently combined with long-acting antimuscarinic agents like tiotropium bromide or/and steroids producing respiratory benefits and better patient survival but signaling pathways involved in inflammatory processes in COPD and inflammatory response to the therapy are not known. We have previously shown that that in cells isolated from induced sputum of COPD patients subjecting to add-on tiotropium therapy acetylated H3 histone levels are significantly higher (Holownia et al. 2010). A similar increase was observed in our patients in AcH4. It appears that histone acetylation/deacetylation balance in tiotropium-treated patients is shifted toward hyperacetylation. Histones are important in inflammatory signaling since they are responsible for gene transcription within chromatin. Histone acetylation mediated by HAT neutralizes the positive charge on the histone molecules (Khan and Khan 2010). As a consequence, chromatin is transformed into a more relaxed structure, associated with greater levels of gene transcription. Several signaling molecules including CREB (cyclic AMP response element binding protein) have histone acetyltransferase (HAT) activity (Bedford and

Table 1.1 Respiratory parameters: forced expiratory volume in 1 s (FEV1), inspiratory capacity (IC), and nuclear specific HDAC activity and acetylated histone 4 (AcH4) levels as well as cytosolic iNOS expression in cells isolated from induced sputum of COPD patients before and after add-on tiotropium therapy

	Formoterol (F)	Formoterol + Tiotropium (FT)
FEV1 (L)	1.57 ± 0.09	1.74 ± 0.11*
	53 %	57 %
IC (L)	2.03 ± 0.14	2.26 ± 0.17*
	67 %	74 %
HDAC	100.0 ± 32.4	78.0 ± 25.4*
AcH4	100.0 ± 27.5	165.0 ± 33.3**
iNOS	100.0 ± 34.1	177 ± 51.3**

*$P < 0.05$; **$P < 0.01$ – compared with corresponding data from F-monotherapy

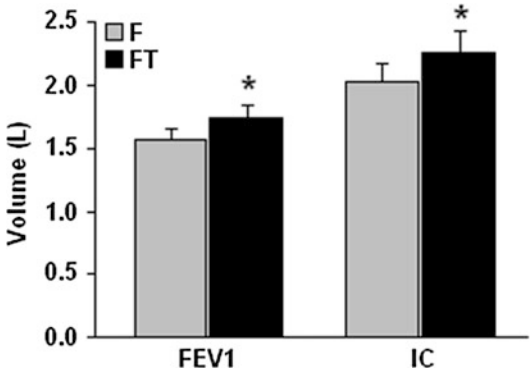

Fig. 1.1 Respiratory parameters: forced expiratory volume in 1 s (FEV1) and inspiratory capacity (IC) in COPD patients before and after 3 months of tiotropium add-on therapy. Tiotropium ameliorated ($P < 0.05$) respiratory parameters of COPD patients and improved their clinical status. *F* formoterol, *FT* formoterol + tiotropium. *$P < 0.05$ – compared with corresponding data from F-monotherapy

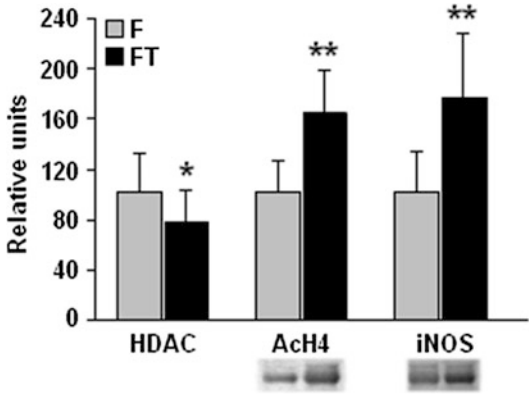

Fig. 1.2 Specific HDAC activity and acetylated histone 4 (AcH4) levels in cell nuclei, and cytosolic iNOS expression in cells isolated from induced sputum of COPD patients before and after 3 months of tiotropium add-on therapy. Representative Western blot pictures of AcH4 and INOS are also shown. *F* formoterol, *FT* formoterol + tiotropium. *$P < 0.05$; **$P < 0.01$ – compared with corresponding data from F-monotherapy

Brindle 2012). HDACs are responsible for removing acetyl groups from histone ε-N-acetyl lysines allowing the histones to tightly wrap the DNA and to limit or even block DNA transcription (Khan and Khan 2010). Several recent rapports stress important role of HDAC in COPD and HDAC activity is clearly decreased in oxidative and/or nitrosative stress (Winkler et al. 2012; To et al. 2012). In asthma bronchial tissue and alveolar macrophages have increased HAT and decreased HDAC (Bouchecareilh and Balch 2012). Our previous reports indicate that HDAC2 expression is not altered after tiotropium therapy (Holownia et al. 2010). Present data indicate however that enzymatic activity of HDAC in cell nuclei is lower. Unfortunately, there is no information on isotype specificity of the substrate which was used in this reaction, but it appears that lower HDAC activity may be responsible, at least in part, for histone hyperacetylation and altered expression of different inflammatory molecules.

In the present study we found increased expression of iNOS after tiotropium therapy. Expression of iNOS may be increased by several factors including inflammatory cytokines, but considering a better clinical status of our patients after tiotropium it seems that this increase is not relevant to pathology. iNOS inhibitors have been shown to block inflammation in a mouse model of COPD (Hesslinger et al. 2009), but were not efficient in asthma (Prado et al. 2011). High nitrosative stress may clearly cause airway inflammation, hyperresponsiveness, and remodeling (Sugiera and Ichinose 2011), while lower NO levels are relevant to airways regulation, especially to smooth muscle relaxation and bronchodilation (Ghosh and Erzurum 2011). We have no data on our IS cellular profiles and we did not assess NO levels which appear to be necessary to explain the reasons and possible roles of increased iNOS. Moreover, there is no published data on the role of histone hyperacetylation in iNOS regulation. However, considering an improvement in clinical status of our patients after tiotropium therapy it seems that increased iNOS expression and altered histone acetylation may have positive consequences.

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

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Yang, S. R., Chida, A. S., Bauter, M. R., Shafiq, N., Seweryniak, K., Maggirwar, S. B., Kilty, I., & Rahman, I. (2006). Cigarette smoke induces proinflammatory cytokine release by activation of NF-kappaB and post-translational modifications of histone deacetylase in macrophages. *American Journal of Physiology - Lung Celllular and Molecular Physiology, 291*, 46–57.

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Involvement of NF-κB and Muscle Specific E3 Ubiquitin Ligase MuRF1 in Cigarette Smoke-Induced Catabolism in C2 Myotubes

2

Sharon Kaisari, Oren Rom, Dror Aizenbud, and Abraham Z. Reznick

Abstract

Cigarette smoking has been identified as a risk factor for muscular damage and sarcopenia, the age-related loss of muscle mass and strength in old age. Cigarette smoke (CS)-induced oxidative stress and p38 MAPK activation have been shown to be the main cellular mechanisms leading to skeletal muscle catabolism. In order to investigate the involvement of NF-κB as another possible cellular mechanism by which CS promotes muscle catabolism, C2 myotubes, from an in vitro skeletal muscle cell line, were exposed to different time periods of whole vapor phase CS in the presence or absence of NF-κB inhibitor, IMD-0354. The CS-induced reduction in diameter of myotubes and time-dependent degradation of the main contractile protein myosin heavy chain were abolished by NF-κB inhibition. Also, C2 exposure to CS resulted in IκB-α degradation and NF-κB activation, which led to upregulation of the muscle specific E3 ubiquitin ligase MuRF1, but not MAFbx/atrogin-1. In conclusion, our results demonstrate that vapor phase CS exposure to skeletal myotubes triggers NF-κB activation leading to skeletal muscle cell damage and breakdown of muscle proteins mediated by muscle specific E3 ubiquitin ligase MuRF1. Our findings provide another possible molecular mechanism for the catabolic effects of CS in skeletal muscle.

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_2,

Keywords
Cigarette smoke • E3 ubiquitin ligases • NF-κB • Muscle atrophy • Protein degradation • p38 MAPK

1 Introduction

Approximately 20 % of the population smoke tobacco worldwide (Basu et al. 2011). Cigarette smoke (CS) is composed of several semi-liquid particles within a mixture of combustion gases (Green and Rodgman 1996). Vapor phase CS consists of aldehydes, nitrogen oxides (NO) and over 1,015 free radicals per puff (Swan and Lessov-Schlaggar 2007) including various reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Smith and Fischer 2011). Smoking is associated with an increased incidence of cardiovascular, cerebrovascular and vascular diseases. Additionally, it is the primary cause of chronic obstructive pulmonary disease (COPD) (Swan and Lessov-Schlaggar 2007). It has been shown that inhalation of both the particulate and vapor phase components of cigarette mainstream smoke lead to pulmonary inflammation in smokers (Rennard and Daughton 1993).

Cigarette smoking has previously been identified as a risk factor for sarcopenia, the loss of skeletal muscle mass and strength in old age (Castillo et al. 2003; Lee et al. 2007; Szulc et al. 2004). Vapor phase CS causes atrophy and degradation of muscle proteins in cultured skeletal myotubes. These catabolic effects are mediated by CS associated activation of p38 mitogen-activated protein kinase (MAPK) and up-regulation of the muscle specific E3 ubiquitin ligases: muscle atrophy F-box protein (MAFbx/atrogin-1) and muscle ring finger-1 protein (MuRF1) which are responsible for determining the exact proteins targeted for proteasomal degradation in skeletal muscle.

Similarly to the p38 MAPK pathway, another major pathway that is activated by CS is the nuclear factor κb (NF-κB) that modulates many vital cellular activities, such as immune, inflammation, survival, and proliferation. Reactive oxygen species (ROS) and TNF-α both activate NF-κB which regulates myogenic activity. Inhibition of the NF-κB pathway in atrophy models prevents muscle degeneration and myofiber death (Meng and Yu 2010). CS has been identified as one of the environmental stimuli that lead to NF-κB activation (Ahn and Aggarwal 2005). Previous work has shown that NF-κB is activated in human lymphocytes exposed to vapor phase CS (Hasnis et al. 2007). In addition, also cigarette smoke condensate (CSC) exposure causes NF-κB activation in various cell lines (Anto et al. 2002).

Activation of NF-κB in muscle-specific transgenic expression of activated IKK (MIKK) mice have been demonstrated to induce significant atrophy through expression of the muscle specific E3 ubiquitin ligase MuRF1, but not MAFbx,/Atrogin-1 (Cai et al. 2004). A signaling pathway of IKK/NF-κB/MuRF1was proposed, in which atrophic stimuli activates NF-κB, which in turn results in upregulation of MuRF1 (Rom et al. 2012). Other findings have also demonstrated that MAFbx/Atrogin-1 upregulation is not required for NF-κB-induced muscle loss (Cai et al. 2004).

The aim of this study was to investigate NF-κB signaling involvement in CS-induced muscle atrophy and degradation of muscle proteins. This was done by exposing C2 myotubes from an in vitro skeletal muscle cell type culture to different levels of whole vapor phase CS in the absence and presence of an NF-κB inhibitor. We assumed that inhibition of NF-κB prior to exposure of cultured myotubes to vapor phase CS will prevent the atrophy of myotubes and protein breakdown through reversed upregulation of the muscle-specific E3 MuRF1, but not MAFbx/atrogin-1.

2 Methods

2.1 Cell Culture

The C2 mouse skeletal myoblast cell line was a generous gift from Prof. E. Bengal (Faculty of Medicine, Technion, Israel). C2 myoblasts were grown in 24 wells, 35 and 100 mm plates in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS), 1 % (v/v) penicillin/streptomycin and 1 % (v/v) L-glutamine at 37 °C in humidified 95 % air-5 % CO2 atmosphere. For the differentiation of myotubes, myoblasts were plated in 0.1 % gelatin-coated plates and were grown to 90 % confluence. At this point, GM was replaced by differentiation medium (DM) consisting of DMEM supplemented with 2 % (v/v) heat-inactivated horse serum, 1 % (v/v) penicillin/streptomycin and 1 % (v/v) L-glutamine. During differentiation DM was replaced every 48 h for 6 days until cell fusion and multi-nucleated myotubes formation was achieved. Successful cell differentiation was determined by expression of the main contractile protein myosin heavy chain (MyHC) as measured by immunoblotting.

2.2 CS Exposure Experiments

Experiments were held on day 7 of differentiation when the cells have completed their differentiation into elongated multi-nucleated myotubes. Exposure to CS was performed by a system consisting of a chamber attached to a vacuum pump and a negative pressure gauge (up to 600 mmHg) at one end and a cigarette at the other end. Myotube plates were placed inside the chamber. Then, the vacuum pump was activated, valve B was closed and valve A was opened until a desired level of negative pressure was created inside the chamber. By using the vacuum pump the pressure inside the chamber was reduced relatively to the atmospheric pressure outside. Subsequently, a TIME commercial cigarette containing 14 mg of tar and 0.9 mg of nicotine and filter (Dubek Ltd., Tel Aviv, Israel) was lit, valve A was closed and valve B between the burning cigarette and chamber was opened for 10 s allowing CS to enter the chamber. Creating reduced pressure inside the chamber allowed the drawing of CS from the burning cigarette into the chamber. Thus, the level on negative pressure inside the chamber equated the dose of CS entering the chamber. Smoke passing through the cigarette filter was considered as vapor phase CS. After exposure to CS, the chamber with the myotube plates was sealed and transferred for different incubation times at 37 °C. Sham-air exposed myotube plates were used as control. In the experiments that examined the effects of CS in increasing incubation periods, control plates were subjected to the most prolonged incubation time with exposure to air instead of CS.

2.3 Measurement of Myotube Diameters

Myotube plates were photographed after CS exposure experiments using a digital camera (Olympus UC30) mounted on a phase contrast microscope (Olympus CK40-SLP) at ×20 magnification. Following experiments, nine fields of view were chosen randomly and ten largest myotubes in each field were measured in a blinded fashion without knowledge of treatment using Image J software. Mean values constituted a measure of 90 myotubes for each experiment. Results were expressed as percent of myotube diameters of the sham-air exposed control plates.

2.4 Cell Lysates Preparation and Western Blot Analysis

Following the CS exposure experiments, cells were washed twice by PBS and lysed for cytosolic proteins using 400 μl/plate lysis buffer consisting of 50 mM Tris HCl pH 7.4, 300 mM NaCl, 1.5 mM $MgCl_2$, 200 mM EDTA and 0.1 % Triton ×100. Protease inhibitor, diluted 40×, and phosphatase inhibitor cocktails (Sigma-Aldrich,

St. Louis, MO) were added to lysis buffer just prior to use. Cells were scraped and transferred to micro-centrifuge tubes for incubation on ice for 10 min followed by centrifugation at 4 °C and 14,000 RPM for 10 min. Supernatants containing cytosolic proteins were collected and kept at −80 °C. Total protein concentrations were measured by Bradford assay (Bio-Rad) using bovine serum albumin as standard. A total protein of 20 μg/lane was loaded and separated by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following SDS-PAGE, proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5 % non-fat milk powder in TBS-T (0.125 % Tween) for 1 h and exposed overnight to primary antibody at 4 °C. Primary antibodies against the following proteins were used: MyHC (1:1,000), MAFbx/atrogin-1 (1:1,000), MuRF1 (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA), actin (1:4,000) (Chemicon International; Temecula, CA), p38 mitogen-activated protein kinase (MAPK) (1:1,000), phospho-p38 MAPK (1:1,000) (R&D Systems; Minneapolis, MN). The next day, membranes were washed with TBS-T followed by 1 h incubation at ambient temperature with appropriate secondary antibodies conjugated to horse-radish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Detection was performed by enzyme-linked chemiluminescence (ECL) using ImageQuant LAS 4,000 digital imager system (GE Healthcare Life Sciences; Rehovot, Israel). Protein quantities were determined by densitometry and analyzed using Total Lab Software.

2.5 Protein Loading Control – The Ponceau S Staining Technique

Since one of our objectives was to examine the effects of CS on actin protein, this protein could not be used as an internal control for protein loading because it was degraded by our treatments. Therefore, we used a quantitation of total proteins by the Ponceau staining before antibody probing as an alternative to housekeeping protein blotting. Romero-Calvo et al. (2010) have shown that reversible Ponceau S staining can be used advantageously over actin detection for equal loading control in Western blotting. Ponceau S is a non-specific protein dye; all proteins in the membrane are colored. After transfer of proteins to nitrocellulose membranes, the membranes were rinsed in Ponceau S solution (Bio-Rad) for 10 min, followed by a brief rinse in double-distilled water (DDW), so that the lanes and bands were clearly visible. Membranes were then inserted in-between transparency sheets and scanned using a standard scanner. Total protein quantity in each lane was determined by densitometry of the scanned membrane using Total Lab Software and used for normalization. At each lane, ECL detected proteins were quantified relatively to total protein quantification found by densitometry of Ponceau S staining. Subsequently, membranes were rinsed once more in DDW until the staining was completely eliminated. From that point on, the blocking and antibody incubation steps were continued as usual.

2.6 RNA Purification, Reverse Transcription, and Quantitative Real Time PCR (qPCR)

Purification of total RNA from myotubes was performed by High Pure RNA Isolation Kit (Roche) according to the manufacturer's instruction. RNA concentrations were quantified at 260 nm by a nano-drop spectrophotometer. Samples were diluted to equal concentrations containing 1 μg of RNA. Samples were used to synthesize cDNA with High Capacity cDNA Reveres Transcription Kit (Applied Biosystems; Foster City, CA) using MultiScribe Reverse Transcriptase, RT buffer, 100 mM dNTP mix, RT random primers, RNase inhibitor, and nuclease free H_2O for a final volume of 20 μl.

qPCR was performed using Corbett Rotor-Gene 6,000 (Qiagen; Hilden, Germany) and qPCR SYBR Green ROX Mix (Thermo Fischer Scientific, Waltham MA). Before qPCR, the efficiency of amplification was determined for each primer set. All primer sets were tested for

efficiency >90 % as required for the ΔΔCt relative quantification algorithm. Three microliters of diluted cDNA was used as template; 2 μl of forward and reverse primer mix (2 μM) was added to 5 μl of SYBR Green ROX Mix master. Reactions were performed in a 10 μl reaction volume under the following conditions: Step 1–15 min at 95 °C; Step 2–5 s at 95 °C; Step 3–30 s at 60 °C, with 40 repeats of Steps 2 and 3. For each sample, a value of the threshold cycle (Ct) was calculated using Rotor Gene 6,000 series software based on the time changes in mRNA expression level calculated subsequent to normalization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The abundance of target mRNA relative to GAPDH was determined by the ΔΔCt relative quantification method. Single products and specific melting temperatures were assessed by melting curve. The following primers (Sigma-Aldrich, St. Louis, MO) were designed by PrimerBank database and checked for specificity using BLAST: GAPDH forward: 5′–AGGTCG GTGTGAACGGATTTG–3′ and reverse: 5′–TGT AGACCATGTAGTTGAGGTCA–3′; MAFbx/ atrogin-1 forward: 5′–CAGCTTCGTGAGCGAC CTC–3′ and reverse: 5′–GCAGTCGAGAAGTCC AGTC–3′; MuRF1 forward: 5′–GTGTGAG GTGCCTAC TTGCTC–3′ and reverse: 5′– GCTC AGTCTTCTGTCCTTGGA–3′.

2.7 NF-κB Signaling Inhibition

To investigate the involvement of NF-κB in CS induced catabolism of myotubes, cultures were pretreated with 10 μM IMD-0354 (Sigma-Aldrich, St. Louis, MO), a selective IκB kinase (IKK) inhibitor (Tanaka et al. 2005), 24 h prior to CS exposure. Myotube diameters, MyHC levels, NF-κB activation, and muscle specific E3s expression were determined and compared with myotubes exposed to CS without IMD-0354 pretreatment.

2.8 p38 MAPK Inhibition

To investigate the involvement of p38 MAPK in CS induced catabolism of myotubes, cultures were pretreated with 5 μM SB203580 (Sigma-Aldrich, St. Louis, MO), a specific inhibitor of p38 MAPK (Li et al. 2005), 15 min prior to CS exposure. NF-κB activation was examined and compared with myotubes exposed to CS without SB203580 pretreatment.

2.9 Statistical Analysis

Statistical analysis was performed with a *t*-test and one-way ANOVA followed by Tukey or Dunnett's tests using SPSS Statistics 16 software (SPSS, IBM, Chicago, IL). $p < 0.05$ was considered statistically significant. Results were expressed as means ± SE of three independent experiments.

3 Results

3.1 CS Stimulates NF-κB Signaling Activation

To explore the effects of CS on NF-κB activation, myotubes were exposed to CS at negative pressure level of 50 mmHg followed by incubation at increasing time periods up to 2 h. Afterwards, myotubes were lysed and subjected to Western blot analysis. Following 2 h of CS exposure, maximal IκB phosphorylation, IκB degradation, and a decrease in NF-κB p65 cytoplasmatic protein level were detected, which indicates NF-κB activation (Fig. 2.1a). Furthermore, it was essential to determine whether this activation was directly CS-induced or triggered indirectly by the known p38 MAPK pathway. For that, myotubes were exposed to CS at negative pressure of 50 mmHg in the presence and absence of p38 MAPK inhibitor, SB-203580. Following CS exposure, myotubes were incubated at increasing time periods up to 6 h, according to the delayed activation observed in Fig. 2.1a. Following incubation, myotubes were lysed and subjected to Western blot analysis to examine IκB-α and p65 protein levels. Control myotubes were exposed to air at the same level of negative pressure and incubated for 6 h. Pretreatment with 5 μM, SB-203580 15 min prior to CS exposure prevented CS induced

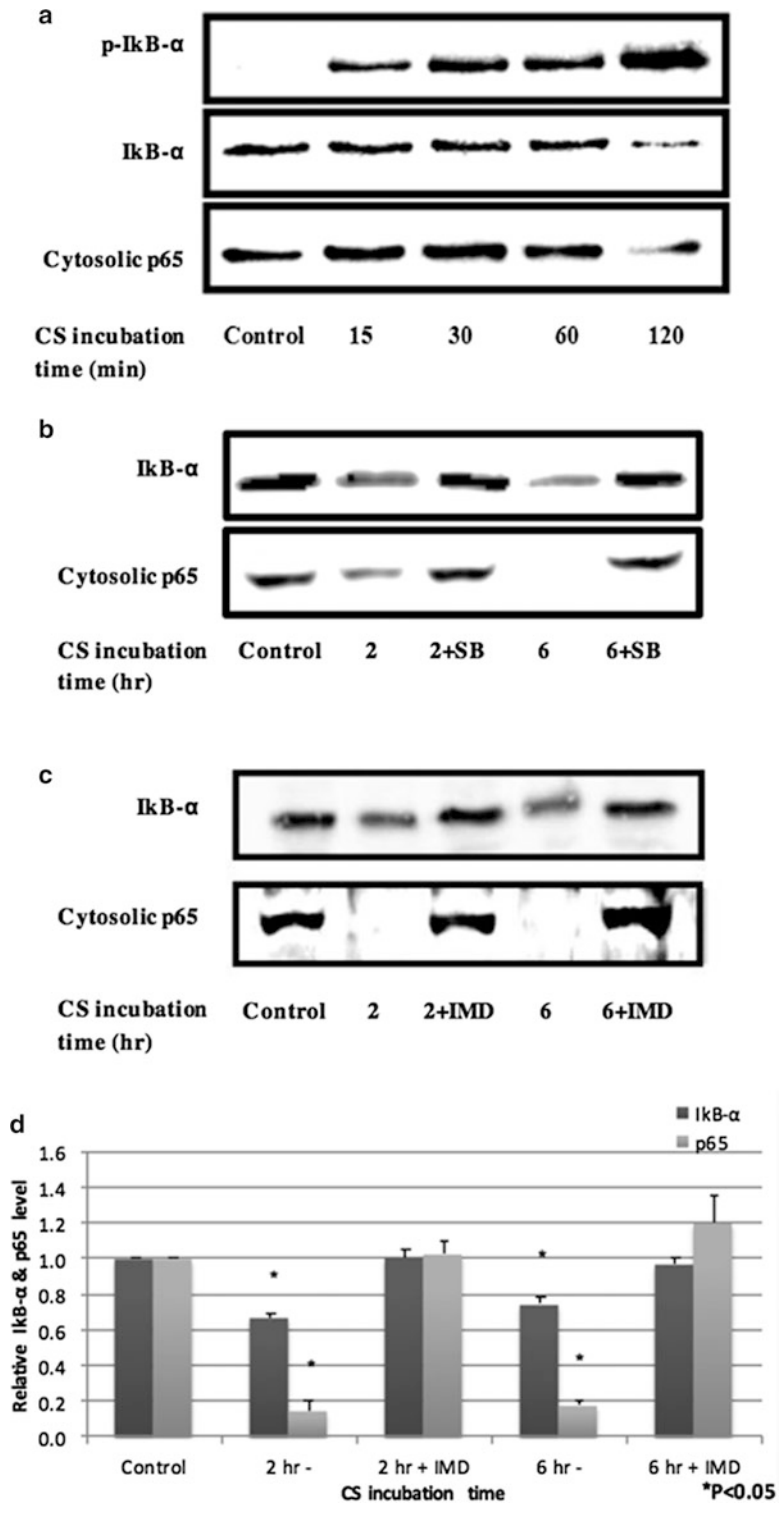

Fig. 2.1 CS exposure activates NF-κB activation via IκB-α degradation and p65 translocation from the cytoplasm into the nucleus in C2 myotubes. (**a**) Myotubes were exposed to CS at negative pressure of 50 mmHg and incubated for increasing time periods. Sham-air exposed myotubes served as control. Following incubation with CS, cell lysates were prepared and subjected to Western blot analysis using antibodies against: Phospho-IκB, IκB-α and NF-κB p65 proteins. (**b**) Myotubes were exposed to CS at negative pressure

significant IκB-α degradation and NF-κB p65 translocation from the cytoplasm (Fig. 2.1b). Subsequently, to investigate inhibition of CS-induced activation of NF-κB signaling activation, myotubes were exposed to CS at negative pressure of 50 mmHg in the presence or absence of IKK kinase inhibitor, IMD-0354. Following the described procedure, IκB-α and p65 protein levels were analyzed. A significant decrease in IκB-α and p65 protein were observed after 2 and 6 h of CS incubation. Pretreatment with 10 μM of IMD-0354. 24 h prior to CS exposure prevented CS induced IκB-α degradation and NF-κB p65 translocation from the cytoplasm (Fig. 2.1c, d).

To examine the role of NF-κB signaling in CS-induced skeletal muscle catabolism, myotubes were exposed to CS at negative pressure of 50 mmHg followed by 6 h of incubation in the absence or presence of 10 μM of IMD-0354, 24 h before exposure. Control myotubes were exposed to air at the same level of negative pressure and incubated for 6 h. Then, myotubes were lysed and subjected to Western blot analysis. MyHC levels decreased significantly following 6 h of incubation with CS in the absence of IMD. Nevertheless, MyHC levels did not change significantly in the presence of IMD. As expected, actin levels did not decrease significantly at any treatment (Fig. 2.2d, f).

3.2 NF-κB Signaling Inhibition Prevents CS Induced Reduction in Myotubes Diameter Breakdown of MyHC

To evaluate NF-κB signaling involvement in CS induced catabolism of skeletal muscle, myotubes were exposed to CS at negative pressure level of 50 mmHg for 6 h in the absence or presence of 10 μM of the NF-κB inhibitor IMD-0354, 24 h before exposure. Control myotubes were exposed to air at 50 mmHg for 6 h. Following incubation, myotubes were photographed and diameters were measured as described in the methods. The myotube diameter decreased depending on the CS incubation time. A significant reduction in myotube diameters was found following 6 h of incubation with CS in the absence of IMD. However, IMD prevented this decrease in myotube diameters following the same incubation protocols (Fig. 2.2a–c, e).

3.3 NF-κB Is Involved in CS-Induced Upregulation of MuRF1

To examine the involvement of NF-κB in CS-induced upregulation of E3s MAFbx/atrogin-1 and MuRF1 in skeletal myotubes, myotubes were exposed to CS at negative pressure of 50 mmHg and incubated for increasing time periods in the absence and presence of 10 μM IMD-0354, 24 h before exposure. Control myotubes were exposed to air at the same level of negative pressure and incubated for 3 h. Following incubation, RNA purification, reverse transcription and qPCR were performed as described in the methods. A significant increase in MAFbx/atrogin-1 and MuRF1 mRNA levels was detected following 1 and 3 h of incubation with CS at 50 mmHg. However, no significant upregulation in MuRF1 mRNA was shown following pretreatment with IMD-0354 whereas in MAFbx/atrogin-1 the up-regulation persisted

Fig. 2.1 (continued) of 50 mmHg and incubated for increasing time periods in the presence or absence of 5 μM of p38 MAPK inhibitor SB-203580. Sham air exposed myotubes served as control. Following incubation with CS, cell lysates were prepared and subjected to Western blot analysis using antibodies against p65 and IκB-α proteins. (**c**) Myotubes were exposed to CS at negative pressure of 50 mmHg and incubated for increasing time periods in the presence or absence of 10 μM of NF-κB inhibitor IMD-0354. Sham air exposed myotubes served as control. Following incubation with CS, cell lysates were prepared and subjected to Western blot analysis using antibodies against p65 and IκB-α proteins. (**d**) p65 and IκB-α protein levels were normalized by total protein densitometry detected by Ponceau S staining and expressed relative to the corresponding value of sham-air exposed control myotubes. Results are expressed as means ± SE of three different experiments. *$p < 0.05$ vs. control myotubes

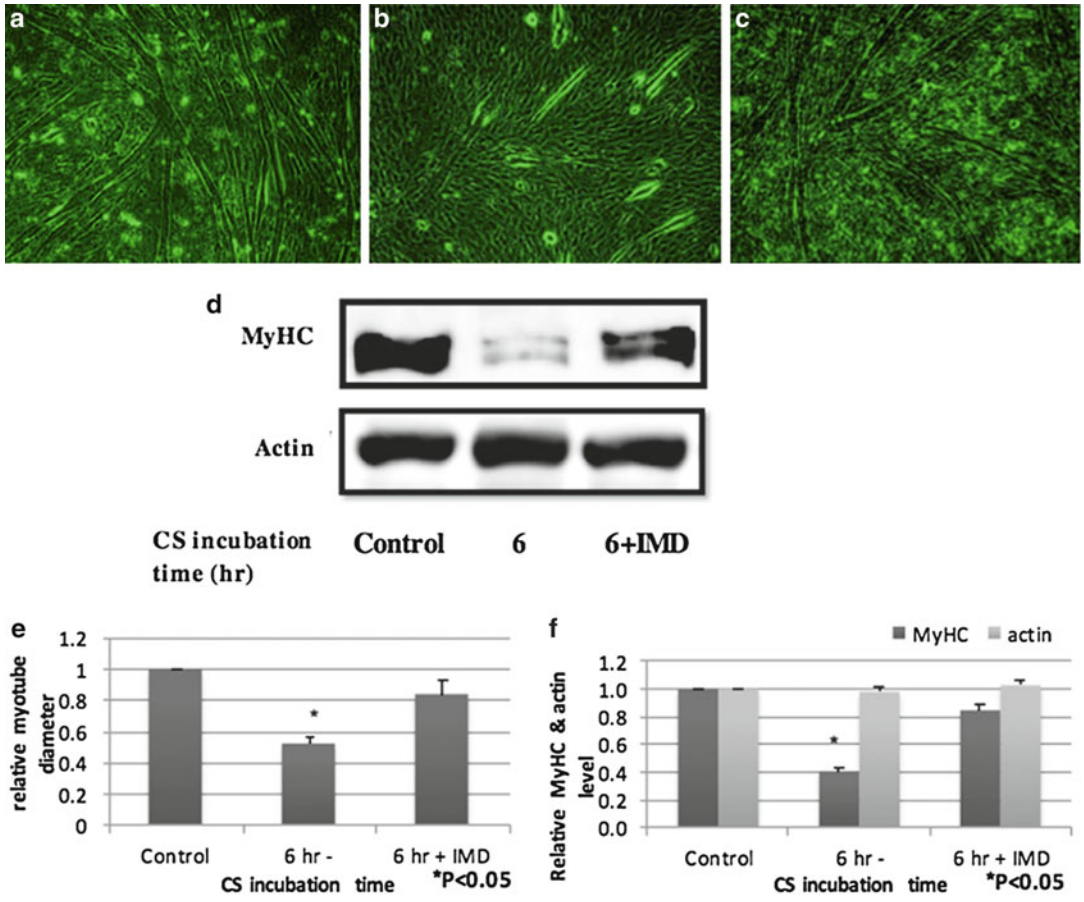

Fig. 2.2 NF-κB inhibition prevents CS-induced reduction in diameter and degradation of MyHC in C2 myotubes. Myotubes were exposed to CS at negative pressure of 50 mmHg and incubated for 6 h in the presence or absence of 10 μM of NF-κB inhibitor IMD-0354 (IMD). Myotubes were photographed at ×20 magnification following increasing incubation time with CS: (**a**) Control (Sham air exposure for 6 h), (**b**) 6 h with IMD, (**c**) 6 h. (**d**) Following incubation with CS, cell lysates were prepared and subjected to Western blot analysis using antibodies against MyHC and actin proteins. (**e**) Changes in myotube diameters are expressed as percent of the diameter in sham-air control myotubes. Results are relative to control and expressed as means ± + SE of three different experiments. *$p < 0.05$ vs. control myotubes. (**f**) MyHC and actin protein levels were normalized by total protein densitometry detected by Ponceau S staining and expressed relative to the corresponding value of sham-air exposed control myotubes. Results are relative to control and expressed as mean ± SE of three different experiments. *$p < 0.05$ versus control myotubes

(Fig. 2.3a). Furthermore, to examine the effects of CS exposure on MAFbx/atrogin-1 and MuRF1 protein levels, myotubes were exposed to CS at 50 mmHg for increasing incubation time periods in the absence or presence of IMD-0354. Control myotubes were exposed to air at the same level of negative pressure and incubated for 6 h. Protein levels were examined by Western blot using appropriate antibodies. Pretreatment with IMD-0354 inhibited the MuRF1 activation, as opposed to the lasting MAFbx/atrogin-1 activation (Fig. 2.3b, c).

4 Discussion

CS is known to be one of the environmental stimuli that causes NF-κB activation (Ahn and Aggarwal 2005). Thus, it was interesting to explore the effects of CS on NF-κB activation in

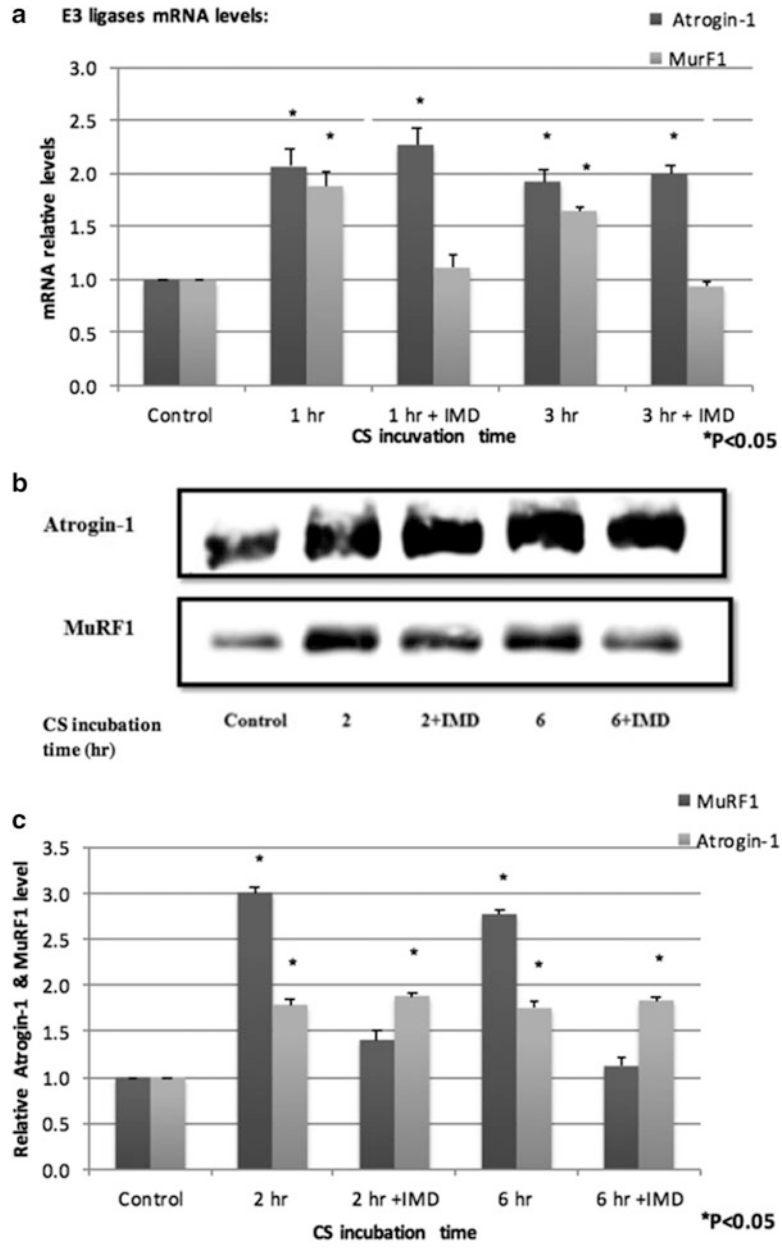

Fig 2.3 NF-κB inhibition prevents CS-induced upregulation of MuRF1, but not that of MAFbx/atrogin-1. (**a**) Myotubes were exposed to CS at negative pressure of 50 mmHg in the presence or absence of 10 μM of NF-κB inhibitor IMD-0354 (IMD) and incubated for increasing time periods. Sham-air exposed myotubes served as control. Following incubation with CS, total RNA was isolated and subjected to reverse transcription and qPCR analysis to determine the expression of MAFbx/atrogin-1 and MuRF1. Data were normalized by GAPDH expression and are relative to the corresponding value of sham air exposed control myotubes. (**b**) Following incubation with CS cell lysated were prepared and subjected to Western blot analysis using antibodies against MAFbx/atrogin-1 and MuRF1 proteins. (**c**) Proteins level were normalized by total protein densitometry detected by Ponceau S staining and expressed relative to the corresponding value of sham-air exposed control myotubes. Results are expressed as means ± E of three different experiments. *p < 0.05 vs. control myotubes

muscle myotubes. In a recent study, we have shown that the first step of CS induced muscle catabolism is oxidative stress (Hasnis et al. 2007). Consequently, p38 MAPK phosphorylation was triggered, which resulted in upregulation of muscle specific E3s. In the current study, it was found that CS stimulates IκB degradation and p65 translocation from the cytoplasm into the nucleus.

Recently, it has been shown that CS exposure causes MyHC degradation in skeletal myotubes

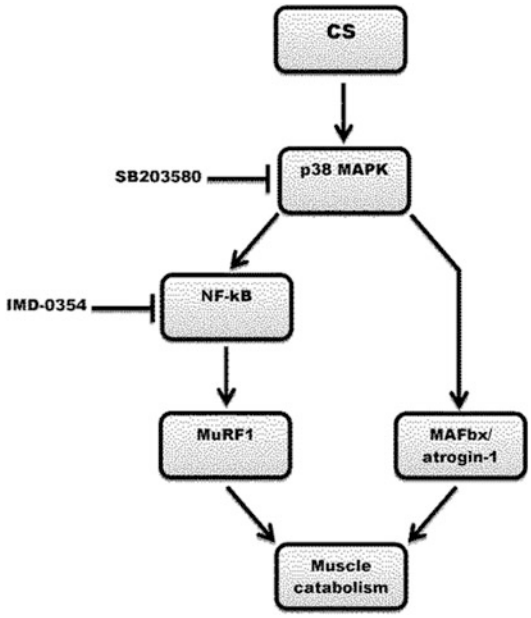

Fig. 2.4 Induction of muscle specific E3 ligases by CS

in vitro (Liu et al. 2011). In the present study, the involvement of NF-κB activation in catabolic effects of C2 myotubes exposure to CS was explored by examining changes in myotube diameters and levels of the most important contractile proteins, MyHC, and actin in the absence or presence of NF-κB inhibitor IMD-0354. Our study demonstrate that prolonged CS exposure resulted in MyHC degradation and reduction in myotube diameters. Furthermore, NF-κB inhibition resulted in a maintained level of MyHC and myotube diameters following prolonged incubation with CS.

Previous studies have shown that exposure of skeletal muscle cells to various ROS and RNS promotes muscle catabolism through activation of the ubiquitin proteasome system (UPS) (Bar-Shai and Reznick 2006) and upregulation of MAFbx/atrogin-1 and MuRF1 (Li et al. 2003). The UPS and the two muscle-specific E3s MAFbx/atrogin-1 and MuRF1 play an important role in the process of muscle wasting degradation (Meng and Yu 2010). Our results demonstrate that upregulation of MuRF1, but not that of MAFbx/atrogin-1 in skeletal myotubes exposed to vapor phase CS was abolished by NF-κB inhibition. These findings demonstrate the role of NF-κB in muscle specific E3 MuRF1 expression and muscle wasting caused by CS exposure. p38 MAPK was previously suggested to trigger the upregulation of MAFbx/Atrogin-1 (Glass 2005; Li et al. 2005). Also, pretreatment with p38 MAPK inhibitor SB203580 blunted the increase in MAFbx/atrogin-1 gene expression. MuRF-1 transcription is believed to be driven by the activation of NF-κB (Cai et al. 2004; Glass 2005; Meng and Yu 2010). In our study, the observed upregulation of MuRF1 following exposure to CS was demonstrated to be a result of direct activation of NF-κB by CS. Another possibility for triggering upregulation of MuRF1 following CS exposure is a biochemical cross-talk between p38 MAPK and the NF-κB pathways. CS activates p38 MAPK, which in turn may activates transcriptional activity of NF-κB leading to MuRF1 upregulation in C2 myotubes (Rom et al. 2012). This possible cross-talk was demonstrated previously by exposing C2C12 myotubes to the inflammatory IL-1 (Li et al. 2009). IL-1 exposure to C2C12 caused p38 MAPK and NF-κB activation resulting in overexpression of both MAFbx/atrogin-1 and MuRF1 leading to loss of myofibrillar proteins and wasting of myotubes (Li et al. 2009).

In conclusion, we show that exposure of vapor phase CS to cultured skeletal myotubes caused atrophy and degradation of muscle proteins via activation of NF-κB pathway and upregulation of MuRF1 in addition to the well known involvement of CS-induced p38 MAPK phosphorylation. Within the limitation of an in vitro study, our findings provide an extra possible molecular mechanism for the complex catabolic effects of CS in skeletal muscle (Fig. 2.4).

Acknowledgments Supported by grants from the Rappaport Institute, the Krol Foundation of Barnegat N. J., the Myers-JDC-Brookdale Institute of Gerontology and Human Development, and ESHEL – the association for planning and development of services for the aged in Israel.

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

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Siglec-8 in Induced Sputum of COPD Patients

3

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Abstract

Chronic obstructive pulmonary disease (COPD) is related to infiltration and activation of inflammatory cells in airways and pulmonary tissue. In COPD, neutrophils are prominent, while eosinophilic influx is typical to asthma. Inflammatory cells express sialic acid-binding immunoglobulin like lectins called Siglecs, a family of innate immune receptors that are transmembrane I-type lectins binding sialic acid. One member of the Siglec family, Siglec-8, is expressed mostly in eosinophils and may be an important therapeutic target in asthma or COPD. The aim of our project was to quantify Siglec-8- expression in induced sputum cells of COPD patients treated with long-acting beta2-agonists (LABA) or combined with long-acting antimuscarinic agents (LAMA) – tiotropium bromide. Thirty stable COPD patients (21 males and 9 females, mean age 67 years) receiving 12 μg BID formoterol therapy were assessed before and after 3 months' add-on therapy consisting of 18 μg QID tiotropium. In all patients, spirometry, lung volumes, and DLCO were performed before and after therapy. The patients were subjected to sputum induction before and after therapy. Sputum cells were isolated and processed to obtain cell membranes. Siglec-8 protein expression was assessed using Western blot. In patients receiving tiotropium and formoterol, improved FEV1 and lung volumes were observed compared with formoterol-only treated patients. The mean Siglec-8 level was significantly higher in eosinophilic subgroup of COPD patients compared with non-eosinophilic patients before therapy 40,000 vs. 15,000 Adj. Vol. INT/mm^2. Our data show that Siglec-8 may be involved in COPD pathogenesis and may influence COPD phenotyping.

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_3,

Keywords
COPD • Siglec-8 • Sputum induction • Tiotropium • Treatment

1 Introduction

According to GOLD (2011) guidelines chronic obstructive pulmonary disease (COPD) is characterized as 'a common preventable and treatable disease characterized by persistent airflow limitation that is usually progressive and is associated with enhanced chronic inflammatory response in the airways and the lung to particles and gases'. Chronic inflammation underlying COPD is driven by macrophages, neutrophiles, cytotoxic T lymphocytes and inflammatory mediators (Mroz et al. 2007). In the minority of COPD patients, eosinophilic involvement, a hallmark of asthma and other allergic disorders, may be found. The sialic acid-binding immunoglobulin like lectins (Siglecs) comprise a family of a single-pass transmembrane proteins that can be characterized by their specificity for sialic acids attached to the terminal regions of cell-surface glycoconjugates (Varki 1992). These innate immune receptors are expressed on inflammatory cells, including pulmonary macrophages, and eosinophils. The molecular mechanisms underlying the recruitment of these cells to sites of inflammation are poorly understood and Siglecs may play an important role in this process. One member of Siglec family, Siglec-8, expressed mostly in eosinophils may be an important therapeutic target in asthma and COPD (Kikly et al. 2000). In the present study, we quantified Siglec-8 expression in induced sputum cells of COPD patients treated with long-acting beta2-agonists (LABA) or combined with a long-acting antimuscarinic agent (LAMA), tiotropium bromide.

2 Methods

2.1 Subjects and Treatment

Thirty patients included into the study gave their consent after a full discussion of the nature of the study, which had been approved by a local Ethics Committee. All patients were stable COPD patients (21 males and 9 females, mean age 67 years) and were characterized with respect to sex, age, smoking history, COPD symptoms, comorbidity, and current medical treatment. The patients had stable disease, defined according to GOLD (2011) guidelines, including airflow limitation (FEV1 < 80 % predicted, FEV1/FVC < 70 %, GOLD stage 2–4). No patient in the study had symptoms nor was treated for COPD exacerbation during at least 2 months before the day of inclusion. Exclusion criteria included other systemic diseases, other lung diseases apart from COPD and lung tumors, pulmonary infections, and antibiotic treatment 4 week before inclusion or inhaled/oral glucocorticosteroids in the 3 months before inclusion. Spirometry and lung volumes were performed with a body box (Elite DL, Medgraphics, USA), using standard protocols.

All patients underwent a 4-week washout therapy with Salbutamol. After that, they were treated for another 4 weeks with 12 μg BID formoterol and then subjected to the sputum induction. Subsequently, the patients were treated for 3 months with add-on 18 μg QID Tiotropium and their sputum was collected again.

2.2 Sputum Induction and Processing

Sputum was induced by the inhalation of a 4.5 % hypertonic aerosol saline solution, generated by an ultrasonic nebulizer (Voyager, Secura Nova; Warsaw, Poland). Three flow volume curves were performed before and after each inhalation, and the best FEV1 was recorded. Induction of sputum was stopped if the FEV1 value fell by at least 20 % from baseline or if troublesome symptoms occurred. Samples were processed within about 15 min after the termination of the induction. Samples were solubilized in equal volumes of 0.1 % dithiothreitol (Sigma Chemicals,

Poznan, Poland) in Hanks solution, and incubated for 15 min in an ice bath. Cell suspension was then rinsed twice with Hanks solution, filtered by a nylon membrane and centrifuged (1,000 rpm) on Histopaque 1,077. Isolated cells were homogenized in a lysis buffer containing 10 mM N-2-hydroxyethylpiperazine-N′-ethane sulfonic acid, 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM ethylenediamine-tetraacetic acid, 0.2 mM NaF, 50 mM β-glycerophosphate, a protease inhibitor tablet, 0.2 mM Na-orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 10 % Nonidet P-40. Thereafter, 50 % of each sample was retained and further processed for other analyses, while retaining sample portion was subjected for western blot analysis. Siglec-8 protein expression was assessed by SDS-PAGE/WB. For Western blots, 15 μg of isolated soluble proteins were separated by SDS/PAGE in reducing conditions, transferred onto polyvinylidene difluoride (PVDF) membranes, and incubated with rabbit antibodies against human Siglec-8. After washing, the bound antibody was detected using an anti-rabbit antibody (Abcam, Cambridge, UK) linked to horseradish peroxidase, and the bound complexes were detected using enhanced chemiluminescence (ECL, Amersham, Little Chalfont, Buckinghamshire, UK) and quantified using Image Quant software. The constitutively expressed protein, b-actin, served as a loading control, and the data were quantified in respect to b-actin expression. Protein levels were measured using a BCA kit (Sigma-Aldrich, Poznan, Poland).

Statistical analysis was performed using statistical package – Statistica (Statsoft, Cracow, Poland) with a nonparametric Wilcoxon test for paired data. The data were expressed as means ± SD. $P < 0.05$ was as considered statistically significant.

3 Results

The patients were stratified according to the content of eosinophils in induced sputum: those with the percentage of eosinophils ≤2 were considered the 'non-eosinophilic' group, and those with the percentage of eosinophils ≥2 were considered the 'eosinophilic' group. The stratification provided 19 non-eosinophilic and 11 eosinophilic patients, and the highest percentage of eosinophils reached was 6 (data not shown). In the patients receiving formoterol and tiotropium, FEV1 and lung volumes were improved compared with the formoterol-only treated patients, regardless of the content of eosinophils (Fig. 3.1a, b). The mean Siglec-8 level was significantly lower before the add-on tiotropium therapy in the non-eosinophilic subgroup of COPD patients (Fig. 3.1a) compared with the eosinophilic subgroup (Fig. 3.2b) 15,000 vs. 40,000 Adj. Vol. INT*mm^2. Furthermore, there were significant changes in response to the add-on therapy applied. The addition of tiotropium increased the mean Siglec-8 level significantly in both subgroups of COPD patients: 26,000 vs. 50,000 Adj. Vol. INT*mm^2 in non-eosinophilic and eosinophilic groups, respectively (Fig. 3.2a, b).

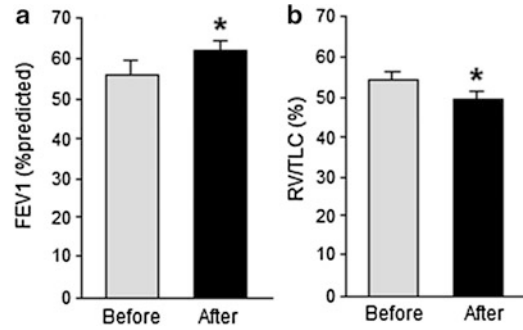

Fig. 3.1 (**a**) Forced expiratory volume in 1 s (FEV1) and (**b**) Ratio of residual volume/total lung capacity (RV/TLC) in COPD patients before and after a 3-month add-on tiotropium therapy. Tiotropium improved respiratory variables. *$p < 0.05$ for the difference from the corresponding data before therapy

4 Discussion

Siglecs are found on different cell types and distinct signaling functions are proposed for these sialic acid-binding immunoglobulins as based on their type (Varki and Gagneux 2012; Varki 1992). Whereas MAG is found exclusively in the nervous system, CD22 only on mature B

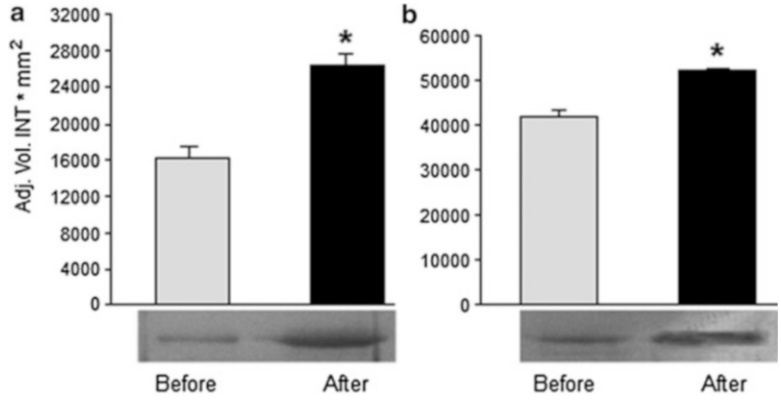

Fig. 3.2 Siglec-8 level in cells isolated from induced sputum in COPD patients before and after a 3-month add-on tiotropium therapy. (**a**) 'Non-eosinophilic group' and (**b**) 'Eosinophilic group'. Representative Western blots of Siglec-8 also are depicted. *$p < 0.05$ for the difference from the corresponding data before treatment

cells, and sialoadhesin is present on CD33+ macrophages, the early committed myeloid progenitor cells. Siglec-5 is characteristic for monocytes and mature neutrophils, Siglec-6 for B cells, and Siglec-7 for NK cells and monocytes (Varki 1992). Recently, several types Siglecs and Siglec-dependent pathways were described. These molecules may play a role in cell signaling in diseases in which the cell subsets above outlined are involved (Varki and Crocker 2009). Siglec-8 which is present on eosinophils, basophiles, and mast cells has gained much of interest lately. The role of eosinophils is well established in asthma, COPD exacerbations, and the overlap syndrome (Bafadhel et al. 2012; Kitaguchi et al. 2012; Rosenberg et al. 2013). Thus, eosinophils appear to be a natural target for treatment. While eosinophiles are the hallmark of asthma, their role in COPD is less elucidated and, if present, rather points to an asthma-COPD overlap syndrome than COPD stand alone (Soler-Cataluna et al. 2012).

In the present study, we found eosinophils in induced sputum in the majority of patients, using a cut-off value of 2 % points. Kitaguchi et al. (2012) have suggested that the cut-off value for the eosinophilic content of 2.5 % would indicate the asthma-COPD overlap syndrome. Irrespective of the content of eosinophils, however, we found Siglec-8 present in all COPD subjects which has not been described previously. The 'eosinophilic group' of patients in the present study was characterized by a higher of Siglec-8 level, but the add-on treatment with tiotropium increased Siglec-8 in both 'eosinophilic' and 'non-eosinophilic' subgroups of COPD patients. According to the recent data, Siglec-8 is considered to be involved in the induction of apoptosis of related cell subsets (Cao and Crocker 2011; Varki and Varki 2007). It is thus possible that treatment which leads to an enhancement of Siglec-related apoptosis may have a role in disease regulation, by downregulating eosinophils and other Siglec-related cell populations. This phenomenon might be relevant for asthma. However, we also observed it in all COPD patients; the finding that may have a meaning in regard to the ongoing debate on the COPD phenotypes and COPD patients with asthmatic symptoms, i.e., the overlap syndrome. It is postulated that in this syndrome inhaled corticosteroids (ICS) should be considered earlier as a potential treatment and a high sputum eosinophil count should be considered as one of the predictors of the response to ICS (Kitaguchi et al. 2012). We did not use ICS in the present series of patients and tiotropium was the only add-on therapy to formoterol. It seems that previously described antiinflammatory properties of tiotropium may have a role in the antiinflammatory signaling related to Siglec-8, due likely to enhanced apoptosis of targeted cells (Holownia et al. 2009, 2010, 2013). It would be of importance in future studies to assess the role of Siglec in COPD patients also treated with ICS. In conclusion, our data show that Siglec-8 may be involved in the COPD pathogenesis and may influence the COPD phenotyping.

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

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Essential Amino Acid Leucine and Proteasome Inhibitor MG132 Attenuate Cigarette Smoke Induced Catabolism in C2 Myotubes

4

Oren Rom, Sharon Kaisari, Dror Aizenbud, and A.Z. Reznick

Abstract

Exposure to cigarette smoke (CS) and cigarette smoking have been shown to promote catabolism of skeletal muscle. Previous studies and recent findings from our laboratory have demonstrated the involvement of the ubiquitin proteasome system and the muscle-specific E3 ubiquitin ligases MAFbx/atrogin-1 and MuRF1 in CS induced skeletal muscle catabolism. The essential amino acid leucine is a known anticatabolic agent that improves skeletal muscle metabolism in various atrophic conditions. To examine the protective effect of leucine and proteasome inhibition in CS induced muscle catabolism, C2 myotubes, from an *in vitro* skeletal muscle cell line, were exposed to CS in the presence or absence of leucine and a proteasome inhibitor, MG132. Diameter of myotubes, levels of the main contractile proteins – myosin heavy chain and actin, expression of MAFbx/atrogin-1 and MuRF1 were studied by microscopy, Western blotting, and qPCR. Leucine pretreatment prevented the CS-induced reduction in diameter of myotubes and degradation of myosin heavy chain by suppressing the upregulation of MAFbx/atrogin-1 and MuRF1. MG132 also attenuated the CS-induced decrease in diameter of myotubes and degradation of myosin

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_4,

heavy chain. Our findings demonstrate that supplementation with the essential amino acid leucine and inhibition of the proteasome may protect skeletal muscle from CS induced catabolism.

Keywords
Cigarette smoke • Leucine • MAFbx/atrogin-1 • MuRF1 • Muscle catabolism • Proteasome

1 Introduction

Exposure to cigarette smoke (CS) and cigarette smoking have been shown to promote catabolism of skeletal muscle in clinical, *in vivo* and *in vitro* studies (Rom et al. 2012). In addition, epidemiological studies have identified smoking as risk factor for sarcopenia, the loss of muscle mass, strength and function in old age (Lee et al. 2007; Szulc et al. 2004; Castillo et al. 2003). CS is a complex aerosol containing over than 4,700 constituents, some of which have the potential to increase catabolism of skeletal muscle. These CS components include reactive oxygen species (ROS), reactive nitrogen species (RNS), and various aldehydes which can damage skeletal muscle by increasing oxidative and nitrative stress and activating muscle specific proteolytic pathways. Based on previous studies and recent findings from our laboratory, we have proposed a cellular model of CS induced catabolism of skeletal muscle (Rom et al. 2012). In this model, components of CS may reach skeletal muscle of smokers, increasing intracellular oxidative stress and activating the p38 mitogen-activated protein kinase (MAPK) and nuclear factor kappaB (NF-κB) pathways. Activation of these pathways results in upregulation of muscle-specific E3 ubiquitin ligases (E3s) of the ubiquitin proteasome system (UPS), leading to increased degradation of muscle proteins and muscle catabolism (Rom et al. 2012).

The UPS is the primary pathway of intracellular protein degradation in skeletal muscle (Foletta et al. 2011). In this pathway 3 major enzymes are involved: E1 ubiquitin activating enzymes, E2 ubiquitin carriers, and E3s which mediate ubiquitination of target proteins (Herningtyas et al. 2008). E3s play the important role of determining which proteins are targeted for degradation by the proteasome (Rom et al. 2012). Two muscle-specific E3s have been identified: Muscle atrophy F-box (MAFbx/atrogin-1) and muscle RING finger-1 (MuRF1) (Foletta et al. 2011). Both muscle-specific E3s are considered to play a major role in muscle atrophy. Indeed, knockout mice lacking these E3s are prevented from muscle atrophy (Herningtyas et al. 2008). In a study recently submitted for publication we report that exposure of C2 myotubes, from an *in vitro* skeletal muscle cell line, to CS caused increased muscle catabolism mediated by upregulation of MAFbx/atrogin-1 and MuRF1.

Essential amino acid (EAA) leucine (Leu) stimulates protein synthesis in muscle by regulation of the mammalian target of the rapamycin (mTOR) signaling pathway. Leu stimulates phosphorylation of mTOR, leading to activation of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase (S6K), resulting in increased muscle protein synthesis (Herningtyas et al. 2008). Pretreatment of C2C12 muscle cells with 5 mM Leu suppressed MAFbx/atrogin-1 and MuRF1 upregulation stimulated by starvation. In addition, Baptista et al. (2010) found that Leu supplementation to rats during hind limb immobilization attenuated loss of muscle mass and minimized gene expression of MAFbx/atrogin-1 and MuRF1. Therefore, in this study we aimed to examine the ability of Leu to reduce the catabolic effects of CS in C2 myotubes. In addition, the protective effects of proteasome inhibition were examined by pretreatment of myotubes with the proteasome inhibitor Z-Leu-Leu-Leu-aldehyde (MG132) prior to CS exposure (Kisselev and Goldberg 2001).

2 Methods

2.1 Cell Culture

The C2 mouse skeletal myoblast cell line was a generous gift from Prof. Bengal (Faculty of Medicine, Technion, Israel). C2 myoblasts were grown in 100 mm plates in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS), 1 % (v/v) penicillin/streptomycin and 1 % (v/v) L-glutamine at 37 °C in humidified 95 % air-5 % CO_2 atmosphere. For differentiation to myotubes, myoblasts were plated in 0.1 % gelatin-coated plates and grown to 90 % confluence. At this point, GM was replaced by differentiation medium (DM) consisting of DMEM supplemented with 2 % (v/v) heat-inactivated horse serum, 1 % (v/v) penicillin/streptomycin and 1 % (v/v) L-glutamine. During differentiation DM was replaced every 48 h for 6 days until cell fusion and multi-nucleated myotubes formation was achieved. Successful cell differentiation was determined by expression myosin heavy chain (MyHC) protein as measured by immunoblotting. Cell media and chemicals were purchased from Biological Industries, Israel.

2.2 CS Exposure Experiments

Experiments were held on day seven of differentiation when the cells have completed their differentiation into elongated multi-nucleated myotubes. Exposure to CS was performed by a system consisting of a chamber attached to a vacuum pump and a negative pressure gauge (up to 600 mmHg) at one end and a cigarette at the other end. Myotube plates were placed inside the chamber. Then, the vacuum pump was activated, valve B was closed and valve A opened until a desired level of negative pressure was created inside the chamber. Subsequently, a TIME commercial cigarette containing 14 mg of tar and 0.9 mg of nicotine and filter (Dubek Ltd., Israel) was lit, valve A was closed, and valve B between the burning cigarette and chamber was opened for 10 s, allowing CS to enter the chamber. Creating reduced pressure inside the chamber allowed the drawing of CS from the burning cigarette into the chamber. Thus, the dose of CS entering the chamber equated the levels of negative pressure created inside the chamber. After exposure to CS the chamber with the myotube plates was sealed and transferred for different incubation times at 37 °C. The level of negative pressure used for CS exposure in this study was 50 mmHg. In a study recently submitted for publication, it was found that this level of CS did not cause a significant reduction in viability of myotubes until 24 h of exposure. Sham-air exposed myotubes were used as control. Control plates were subjected to the same incubation periods with exposure to air instead of CS.

2.3 Measurement of Myotube Diameters

Myotube plates were photographed after CS exposure experiments using a digital camera (Olympus UC30, Japan) mounted on a phase contrast microscope (Olympus CK40-SLP, Japan) (objective ×20). Following experiments, nine fields of view were chosen randomly and ten largest myotubes in each field were measured by Image J software (NIH, USA). Measurements were made in a blinded fashion without the knowledge of treatment. The mean values constituted a measure of 90 myotubes for each experiment. Results were expressed as percent of myotube diameters of the sham-air exposed control plates.

2.4 Cell Lysates and Western Blot Analysis

Following the CS exposure experiments, cells were washed twice by PBS and lysed for cytosolic proteins using 400 μl/plate lysis buffer consisting of 50 mM Tris HCl pH 7.4, 300 mM NaCl, 1.5 mM $MgCl_2$, 200 mM EDTA and 0.1 % Triton ×100. ×40 diluted protease inhibitor and

phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO) were added to lysis buffer just prior to use. Cells were scraped and transferred to micro-centrifuge tubes for incubation on ice for 10 min followed by centrifugation at 4 °C and 14,000 RPM for 10 min. Supernatants containing cytosolic proteins were collected and kept at −80 °C. Total protein concentrations were measured by Bradford assay (Bio-Rad, USA) using bovine serum albumin as standard. A total protein of 20 μg/lane was loaded and separated by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5 % non-fat milk powder in TBS-T (0.125 % Tween) (Sigma-Aldrich, St. Louis, MO) for 1 h and exposed overnight to primary antibody at 4 °C. Primary antibodies against MyHC (1:1,000) (Santa Cruz, USA), actin (1:4,000) (Millipore, USA) were used. The next day, membranes were washed with TBS-T followed by 1 h incubation at ambient temperature with appropriate secondary antibodies conjugated to horse-radish peroxidase (Jackson Immuno-Research, USA). Detection was performed by enzyme-linked chemiluminescence (ECL) (Biological Industries, Israel) using ImageQuant LAS 4,000 digital imager system (GE Healthcare, UK). Protein quantities were determined by densitometry and analyzed using Total Lab Software (version V2006C, Nonlinear Dynamics, UK).

2.5 Protein Loading Control by Ponceau S Staining

Actin protein could not be used as an internal control for protein loading because it has been found to be degraded by CS exposure. Therefore, we used quantitation of total proteins by Ponceau staining before antibody probing as an alternative to housekeeping protein blotting. Romero-Calvo et al. (2010) have shown that reversible Ponceau S staining can be used advantageously over actin detection for equal loading control in Western blotting. Ponceau S is a non-specific protein dye; all proteins in the membrane are colored. After transfer of proteins to nitrocellulose membranes, the membranes were rinsed in Ponceau S solution (Bio-Rad, USA) for 10 min, followed by a brief rinse in double-distilled water (DDW) so that the lanes and bands were clearly visible. Membranes were then inserted in between transparency sheets and scanned using a standard scanner. Total protein quantity in each lane was determined by densitometry of the scanned membrane using Total Lab Software (version V2006C, Nonlinear Dynamics, UK), and was used for normalization. At each lane, ECL detected proteins were quantified relatively to total protein quantification found by densitometry of Ponceau S staining. Subsequently, membranes were rinsed once more in DDW until the staining was completely eliminated. From that point, the blocking and antibody incubation steps were continued as usual.

2.6 RNA Purification, Reverse Transcription and qPCR

Purification of total RNA from myotubes was performed by High Pure RNA Isolation Kit (Roche, Germany) according to the manufacturer's instruction. RNA concentrations were quantified at 260 nm by Nanodrop spectrophotometer (Nanodrop Technologies, USA). Samples were diluted to equal concentrations containing 1 μg of RNA. Samples were used to synthesize cDNA with High Capacity cDNA Reveres Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instruction for a final volume of 20 μl.

qPCR was performed using Corbett Rotor-Gene 6,000 (Qiagen, Germany) and qPCR SYBR Green ROX Mix (Thermo Scientific, UK). Before qPCR, the efficiency of amplification was determined for each primer set. All primer sets were tested for efficiency >90 % as required for the ΔΔCt relative quantification algorithm. 3 μl of diluted cDNA were used as template; 2 μl of forward and reverse primer mix (2 μM) were added to 5 μl of SYBR Green ROX Mix master. Reactions were performed in a 10 μl reaction volume under the following conditions:

Step 1–15 min at 95 °C; Step 2–5 s at 95 °C; Step 3–30 s at 60 °C, with 40 repeats of Steps 2 and 3. For each sample, a value of the threshold cycle (Ct) was calculated using Rotor Gene 6,000 series software (Qiagen, Germany) based on the time changes in mRNA expression level calculated subsequent to normalization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The abundance of target mRNA relative to GAPDH was determined by the ΔΔCt relative quantification method. Single products and specific melting temperatures were assessed by melting curve. The following primers (Sigma-Aldrich, St. Louis, MO) were designed by PrimerBank database: GAPDH forward: 5′–AGGTCGGTGTGAACGGATTTG–3′, reverse: 5′–TGTAGACCATGTAGTTGAGGTCA–3′; MAFbx/atrogin-1 forward: 5′–CAGCTTCG-TGAGCGACCTC–3′, reverse: 5′–GCAGT-CGAGAAGTCCAGTC–3′; MuRF1 forward: 5′–GTGTGAGGTGCCTACTTGCTC–3′, reverse: 5′–GCTCAGTCTTCTGTCCTTGGA–3′.

2.7 Leu and MG-132 Pretreatment

To examine the effects of Leu on CS induced muscle catabolism, myotubes were pretreated with 5 mM Leu (Fluka BioChemika, Switzerland) (Herningtyas et al. 2008) 24 h prior to CS exposure. Also, to examine the protective effect of MG132 in CS induced catabolism, myotubes were pretreated with 25 μM MG132 (Enzo, USA) 1 h prior to CS exposure. Diameter of myotubes, MyHC and actin protein levels and expression of muscle specific E3s were examined in CS exposed myotubes in the presence or absence of Leu or MG132.

2.8 Statistical Analysis

Statistical analysis was performed by a *t* test and one-way ANOVA followed by Tukey or Dunnett's test using SPSS Statistics 16 software (IBM, USA). $P < 0.05$ was considered statistically significant. Results were expressed as means ± SE of three independent experiments.

3 Results

3.1 Leu Prevents CS-Induced Myotube Wasting and MyHC Degradation

Recently we have reported that exposure of C2 myotubes to CS caused significant reductions in diameters of myotubes and in the level of MyHC protein (Rom et al. 2012). To examine the ability of Leu to prevent the catabolic effect of CS exposure, C2 myotubes were pretreated with 5 mM Leu 24 h before exposure to CS at 50 mmHg. Diameters and levels of MyHC and actin proteins were examined in myotubes exposed to CS followed by 6 h of incubation and in myotubes pretreated with 5 mM Leu prior to CS exposure. Control myotubes were treated in the same manner with exposure to air instead of CS with or without Leu pretreatment. Air exposed myotubes pretreated with Leu presented greater diameters and MyHC protein level when compared with control myotubes without Leu pretreatment, although these effects were not significant. Diameters of myotubes and MyHC level decreased significantly in the CS-exposed myotubes without Leu pretreatment when compared with the control myotubes. These effects were prevented in the CS-exposed myotubes pretreated with Leu 24 h prior to exposure. The level of actin protein remained stable and did not change under any treatment (Fig. 4.1a–g).

3.2 Leu Attenuates CS-Induced Upregulation of Muscle Specific E3s

Recently we have reported that exposure of C2 myotubes caused a significant increase in mRNA levels of MAFbx/atrogin-1 and MuRF1 (Rom et al. 2012). Also, MAFbx/atrogin-1 and MuRF1 are upregulated in mice chronically exposed to CS (Tang et al. 2010). To examine the effect of Leu on the expression levels of these muscle specific E3s following exposure to CS,

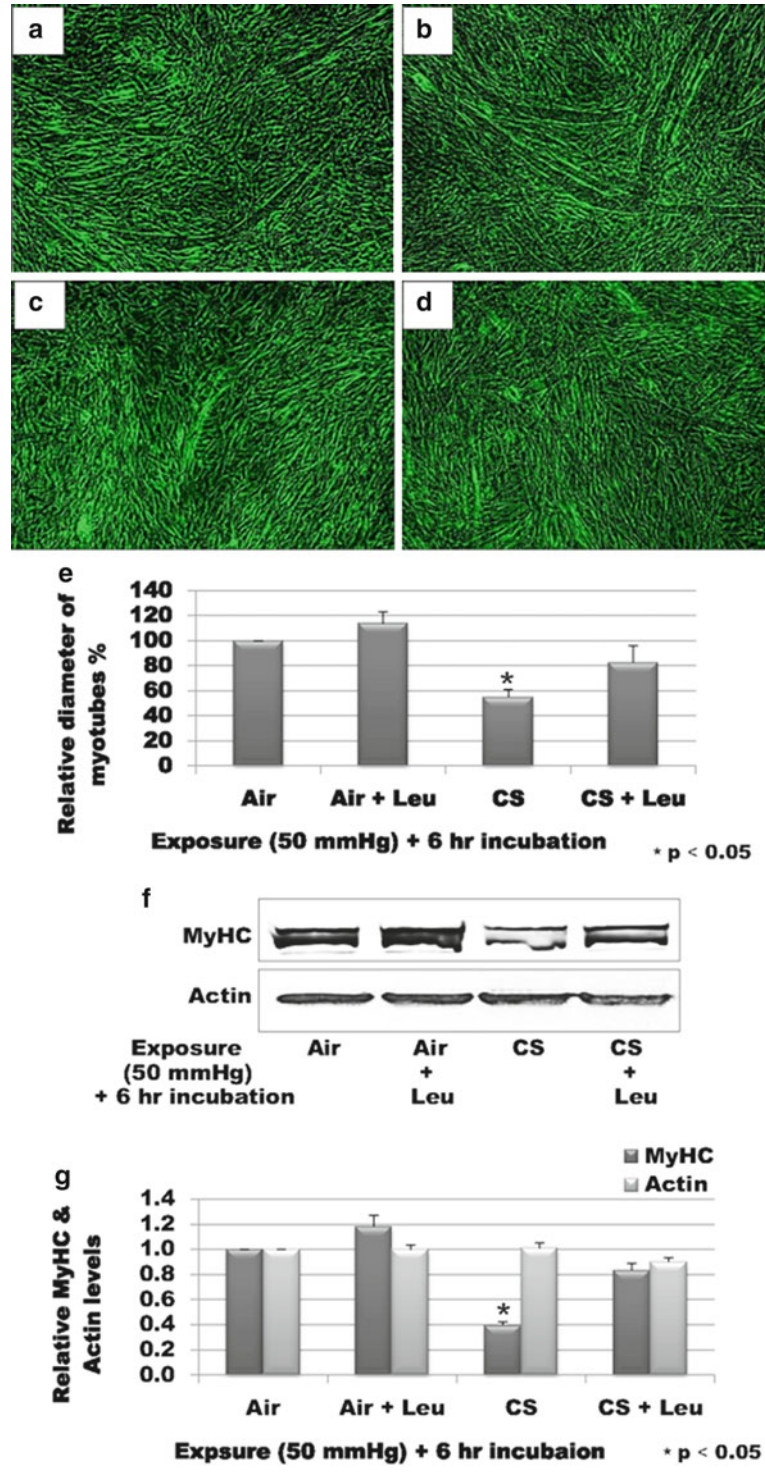

Fig. 4.1 Leu prevents CS induced myotube wasting and MyHC degradation. Myotubes were exposed to CS or air at negative pressure level of 50 mmHg with or without Leu pretreatment followed by incubation for 6 h. Following incubation, myotubes were photographed (×20): (**a**) Control – myotubes exposed to air without Leu pretreatment followed by 6 h incubation. (**b**) Myotubes pretreated with 5 mM Leu 24 h prior to air exposure and 6 h incubation. (**c**) Myotubes exposed to CS without Leu pretreatment followed by 6 h incubation. (**d**) Myotubes pretreated with 5 mM Leu 24 h prior to CS exposure and incubation for 6 h. (**e**) Changes in myotube diameters are expressed as percent of the diameter of control myotubes. Results are relative to control and expressed as means ± SE of three different experiments. Following incubation, cell lysates were prepared and subjected to Western blot analysis using antibodies against MyHC and actin. (**f**) Representative Western blot is presented. (**g**) MyHC and actin protein levels were normalized by total protein densitometry detected by Ponceau S staining and expressed relative to the corresponding value of control myotubes. Results are expressed as means ± SE of three different experiments. *$p < 0.05$ vs. control myotubes

C2 myotubes were pretreated with 5 mM Leu 24 h before exposure to CS at 50 mmHg followed by 3 h of incubation. Following incubation mRNA levels of MAFbx/atrogin-1 and MuRF1 were examined in CS exposed myotubes without Leu pretreatment and in myotubes pretreated

with 5 mM Leu prior to CS exposure. Control myotubes were treated in the same manner with exposure to air instead of CS with or without Leu pretreatment. In air exposed myotubes pretreated with Leu, lower mRNA levels of MAFbx/atrogin-1 and MuRF1 were found, although this was not significant. Compared with the control myotubes, mRNA levels of MAFbx/atrogin-1 and MuRF1 increased significantly in the CS-exposed myotubes without Leu pretreatment. This upregulation was abolished in CS exposed myotubes pretreated with Leu 24 h prior to exposure (Fig. 4.2).

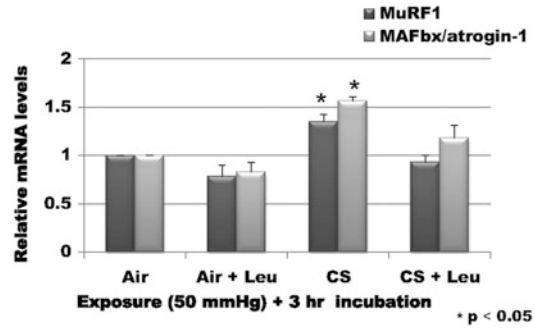

Fig. 4.2 Leu attenuates CS induced upregulation of muscle specific E3s. Myotubes were exposed to CS or air at negative pressure level of 50 mmHg with or without Leu pretreatment followed by incubation for 3 h. Control myotubes were exposed to air without Leu pretreatment. Following incubation, total RNA was isolated and subjected to reverse transcription and qPCR analysis to determine the expression of MAFbx/atrogin-1 and MuRF1. Data were normalized by GAPDH expression and are relative to the corresponding value of control myotubes. Results are expressed as means ± SE of three different experiments. *p < 0.05 vs. control myotubes

3.3 Proteasome Inhibition Prevents CS-Induced Myotube Wasting and MyHC Degradation

To examine the effect of proteasome inhibition on the CS-induced muscle catabolism, C2 myotubes were pretreated with 25 μM MG132 1 h prior to CS exposure. Diameters and levels of MyHC and actin proteins were examined in myotubes exposed to CS in negative pressure of 50 mmHg followed by 6 h of incubation and in myotubes pretreated with MG132 prior to CS exposure. Control myotubes were treated in the same manner with exposure to air instead of CS. Diameters of myotubes and MyHC level decreased significantly in CS exposed myotubes without MG132 pretreatment when compared with air exposed myotubes. These effects were prevented in CS exposed myotubes pretreated with MG132 1 h prior to exposure. The level of actin protein remained stable and did not change under any treatment (Fig. 4.3a–f).

4 Discussion

In this study, we reveal that the catabolic effects of CS were prevented in myotubes pretreated with EAA Leu. Leu pretreatment prior to CS exposure prevented CS induced reduction in myotube diameters and degradation of MyHC. Interestingly, myotubes exposed to air instead of CS and pretreated with Leu, presented greater diameters and higher protein level of MyHC. Although these findings were not significant, they imply that Leu pretreatment increases anabolism of myotubes and thus may promote their resistance to the catabolic effects of CS. Also, Leu pretreatment prevented CS induced upregulation of the muscle specific E3s: MAFbx/atrogin-1 and MuRF1. Since these E3s play a major role in targeting muscle proteins for proteasomal degradation (Rom et al. 2012; Foletta et al. 2011; Herningtyas et al. 2008), prevention of their upregulation may be the key effect of Leu that protects myotubes from CS induced catabolism. These findings are consistent with previous studies reporting that Leu prevents muscle catabolism by suppressing upregulation of these E3s (Baptista et al. 2010; Herningtyas et al. 2008). Leu supplementation is known to improve muscle remodeling in various atrophic states including muscle disuse, sarcopenia, and cancer (Nicastro et al. 2011). Compared with non-smokers, skeletal muscle of smokers presented structural and metabolic damages (Montes de Oca et al. 2008). Lower fractional synthesis rate of muscle and higher expression level of MAFbx/atrogin-1 were found in smokers in comparison with non-smokers (Petersen et al. 2007). Also, chronic CS exposure

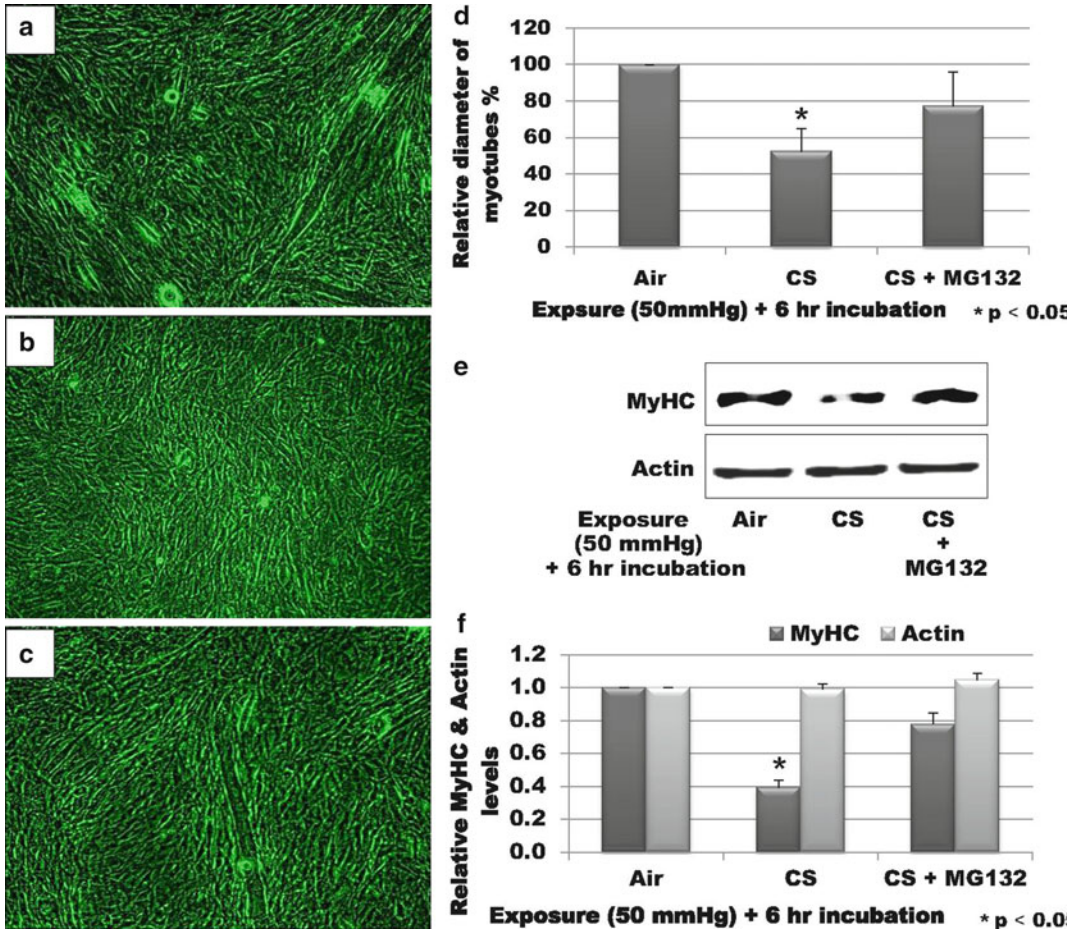

Fig. 4.3 Proteasome inhibition prevents CS induced myotube wasting and MyHC degradation. Myotubes were exposed to CS or air at negative pressure level of 50 mmHg with or without MG132 pretreatment followed by incubation for 6 h. Following incubation, myotubes were photographed (×20): (**a**) Control – myotubes exposed to air without MG132 pretreatment followed by 6 h incubation. (**b**) Myotubes exposed to CS without MG132 pretreatment followed by 6 h incubation. (**c**) Myotubes pretreated with 25 μM MG132 1 h prior to CS exposure and incubation for 6 h. (**d**) Changes in myotube diameters are expressed as percent of the diameter of control myotubes. Results are relative to control and expressed as means ± SE of three different experiments. Following incubation, cell lysates were prepared and subjected to Western blot analysis using antibodies against MyHC and actin. (**e**) Representative Western blot is presented. (**f**) MyHC and actin protein levels were normalized by total protein densitometry detected by Ponceau S staining and expressed relative to the corresponding value of control myotubes. Results are expressed as means ± SE of three different experiments. *$p < 0.05$ versus control myotubes

to mice resulted in loss of skeletal muscle mass and upregulation of MAFbx/atrogin-1 and MuRF1 (Tang et al. 2010). Therefore, we suggest that Leu supplementation may be effective in reducing skeletal muscle catabolism in smokers by suppressing muscle specific E3s. To further establish this, Leu supplementation should be examined in clinical and *in vivo* studies investigating the protective effects of Leu on skeletal muscles of smokers and CS exposed animals.

Our findings also indicate the involvement of the proteasome in the CS-induced muscle catabolism. Pretreatment with the proteasome inhibitor MG132 prior to CS exposure prevented CS induced reduction in myotube diameters and degradation of MyHC. These findings imply that the

UPS is the primary pathway of CS-induced degradation of muscle proteins leading to reduced myotube diameters.

In conclusion, this study demonstrates that EAA Leu attenuates CS induced catabolism of C2 myotubes including reduction of myotube diameters and MyHC degradation. These effects were mediated by attenuating upregulation of the muscle specific E3s: MAFbx/atrogin-1 and MuRF1. Also, inhibition of the proteasome by MG132 prevented CS induced myotube catabolism, demonstrating the involvement of the UPS in CS induced skeletal muscle catabolism.

Acknowledgments This study was supported by grants from the Rappaport Institute, the Krol Foundation of Barnegat N.J., the Myers-JDC-Brookdale Institute of Gerontology and Human Development, and ESHEL – the association for planning and development of services for the aged in Israel.

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

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Smoking Denial in Cardiovascular Disease Studies

5

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Abstract

Assessment of self-reported smoking behavior in cardiovascular studies may lead to inaccurate measures of nicotine exposure. A more objective measurement of nicotine exposure can be done by measurement of plasma cotinine levels. The aim of the present study was to define the rate of discordance between the self-reported smoking behavior and biochemically defined smoking status. Data from 3,316 patients hospitalized for coronary angiography, who completed a questionnaire on smoking behavior, were analyzed. As a biochemical assessment of smoking status we used a cut-off serum cotinine level of 15 μg/l. Smoking denial, defined as a discrepancy between high cotinine levels and self-reported never- or ex-smoking status, was observed in 3.7 % of the study participants. In a logistic regression analysis with a step-wise inclusion of sex, age, CAD, previous MI, and educational level, only male sex (odds ratio male/female: 2.00, 95 % CI 1.22–3.33; $p = 0.007$) and age (odds ratio per year: 0.79, 95 % confidence interval 0.66–0.94, $p = 0.008$) were associated with smoking denial. In conclusion, a misclassification rate of 3.7 % in the evaluation of such an important risk factor may lead to blurred effects and favor false negative results. The results of the present study substantiate the importance of biochemical markers for smoking assessment in cardiovascular studies.

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788, DOI 10.1007/978-94-007-6627-3_5,

Keywords
Cardiovascular disease • Cotinine • Epidemiology • Nicotine exposure • Smoking denial

1 Introduction

Active and passive smoking have been associated with an array of adverse effects on health (Reaven and Tsao 2003; Teo et al. 2006; Jefferis et al. 2010). The development of valid and accurate scales of measurement for exposures associated with health risks constitutes an active area of research. Tobacco smoke exposure still lacks an ideal method of measurement. Assessment of self-reported smoking behavior may lead to inaccurate measures of nicotine exposure; therefore, more objective solutions have been suggested. Biomarkers constitute the most commonly used objective method of ascertaining nicotine exposure. Of those available, cotinine has gained supremacy as the biomarker of choice (Whincup et al. 2004; Bramer and Kallungal 2003; SRNT Subcommittee on Biochemical verification 2002; Benowitz and Jacob 1993).

It is likely that in clinic-based studies, social desirability and expectations of the healthcare team may influence the self-reported smoking behavior among current smokers, who may deny any smoking to appear more compliant. Rates of misclassification of self-reported non-smokers seem to be greater in clinic-based studies compared with population-based studies.

The main aim of the present study was to evaluate the rate of smoking denial, which is a self-reported status of ex- or never-smoking despite being a current smoker, in the Ludwigshafen Risk and Cardiovascular Health (LURIC) study. An additional aim was to define factors associated with smoking denial.

2 Methods

The LURIC study includes consecutive white patients hospitalized for coronary angiography between June 1997 and May 2001. The study was approved by the Ethics Review Committee at the Landesarztekammer Rheinland-Pfalz in Mainz, Germany. Written informed consent was obtained from each participant.

A detailed description of LURIC has been published (Winkelmann et al. 2001). Coronary artery disease (CAD) was defined as the presence of a visible luminal narrowing ($\geq$20 % stenosis) in at least one of 15 coronary segments according to the classification of the American Heart Association (AHA). Additionally, angiographic severity of disease was defined as 0-, 1-, 2- or 3-vessel disease based on the number of luminal narrowing $\geq$50 % in the three major coronary arteries. Previous myocardial infarction (MI) was diagnosed, if there was a conclusive positive history of MI or if patients presented with ST elevation MI. The patients completed questionnaires including detailed questions about current and previous smoking history (cigarettes, pipes, and cigars), other health behaviors, occupation, and medication use.

Fasting blood samples were obtained by venipuncture in the early morning. Serum cotinine was analyzed by a radioimmunoassay (Nikotin-Metabolit RIA, DPC Biermann GmbH, Bad Nauheim, Germany). For serum cotinine we used as cut-off of 15 μg/l, which resulted in the best sensitivity (94.8 %) and specificity (95.6 %) to discriminate smokers from non-smokers (Florescu et al. 2009).

3 Results

All 3,316 study participants completed a questionnaire on smoking behavior. Using this questionnaire, 1,194 (36.0 %) participants reported themselves as never-smokers, 1,468 (44.3 %) as ex-smokers, and 654 (19.7 %) as current smokers.

Using a biochemical assessment of smoking status based upon serum cotinine levels, 2,819 (85.0 %) had serum cotinine levels below 15 μg/l and 497 (15.0 %) had serum cotinine levels

higher than 15 μg/l. In 123 (3.7 %) subjects, cotinine levels did conform to the self-reported smoking behavior, including 107 self-reported ex-smokers and 16 self-reported never-smokers. A discrepancy between high cotinine levels and self-reported never- or ex-smoking status was classified as smoking denial.

To identify potential factors associated with smoking denial, we performed a logistic regression analysis with step-wise inclusion of sex, age, CAD, previous MI, and educational level. Only male sex (odds ratio male/female: 2.00, 95 % CI 1.22–3.33; $p = 0.007$) and age (odds ratio per year: 0.79, 95 % confidence interval 0.66–0.94, $p = 0.008$) were associated with smoking denial.

4 Discussion

In the present epidemiological cardiovascular study that included 3,316 consecutive white patients hospitalized for coronary angiography, 3.7 % who had reported themselves as never- or ex-smoker had serum cotinine levels higher than 15 μg/l, suggesting that they were 'hidden' active smokers.

The assessment of tobacco smoking was achieved through a structured interview. Interviews and questionnaires are the most commonly used vehicle for assessing the use and exposure to tobacco smoke. They are convenient for many reasons: information on exposure can be collected retrospectively, which is of value when data on air pollutant concentrations or biomarkers are not available; they can provide information on long-term exposure; and they are inexpensive to administer to large numbers of subjects and are thus particularly suited for large studies. These questionnaires have been used in many smoking cessation studies (Fergusson et al. 1998; Brooks et al. 2004).

Cotinine, as the major proximate metabolite of nicotine, has become the biomarker of choice for cigarette smoking and environmental tobacco smoke, with specificity (percentage of non-smokers classified as non-smokers) and sensitivity (percentage of smokers classified as smokers) both over 95 %. A cut-off of about 15 ng/ml for either plasma or saliva cotinine to discriminate current smokers from non-smokers has been applied widely (Jarvis et al. 2008). The Office of Environmental Health Hazard Assessment reports at least an order of magnitude difference in cotinine concentrations between active smokers and non-smokers. In that study, unexposed non-smokers had a plasma cotinine concentration of 0.31 ng/ml, whereas exposed non-smokers averaged 1.99 ng/ml (Florescu et al. 2009). It is, therefore, very unlikely that the results of the present study were biased by passive smoking.

It is likely that in clinic-based studies, social desirability and quitting expectations on the part of the healthcare team influence the integrity of the self-report, especially in the case of lighter and occasional smokers, who may deny any smoking to appear compliant. The rates of misclassification of self-reported non-smokers seem to be greater in the clinic-based studies compared with the population-based studies. Among 91 UK patients with oral cancer, 9.6 % of self-reported non-smokers had a salivary cotinine level above 14 ng/ml (Sandhu et al. 2004). Even higher rates were reported in a population of patients with colorectal adenoma from Arizona, in which 20 % of self-reported non-smokers were misclassified based on a serum cotinine cut-off of 20 ng/ml (Martinez et al. 2004).

Misclassification rates for current smokers who report no smoking were calculated by Wells et al. (1998) based on ten large studies that measured cotinine in body fluids and self-reported smoking status in a total of 14,554 subjects. The misclassification rates for female smokers misclassified as never smokers were 1, 6, 3, and 15 % for majority regular smokers, majority occasional smokers, U.S. minority regular smokers, and U.S. minority occasional smokers, respectively. In a population-based study of the U.S. population aged 17 years and older, 1.4 % of self-reported non-smokers had a serum cotinine level above 15 ng/ml, the selected cut-off value for identifying smokers (Caraballo et al. 2001). Similar misclassification rates were found in another U.S. population–based study, where 2.7 % of self-reported non-smokers had serum cotinine above 15 ng/ml (Nondahl et al. 2005). In a Finnish population–based study of 5,846 randomly selected subjects enrolled in smoking cessation programs, the misclassification

rate of those reporting no smoking in the previous month was 5.2 % for women and 6.3 % for men, based on a serum cotinine concentration of above 10 ng/ml (Vartiainen et al. 2002).

Interestingly, in the present study, men were twice as likely to deny smoking as women. The reason for this is not clear, but might be related to differences in health consciousness among men and women. Furthermore, smoking denial was less likely with increasing age. Again, the reason for this is not clear.

Smoking is one of the most prominent risk factors for cardiovascular diseases, comparable to diabetes, dyslipidemia, or hypertension. A misclassification rate of 3.7 % in the evaluation of such an important risk factor may lead to blurred effects and favor false negative results. The results of the present study substantiate the importance of biochemical markers for smoking assessment in cardiovascular studies.

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

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Differential Effects of Kainic Acid Lesions in Medullary Raphe on Cough and Sneeze in Anesthetized Rabbits

6

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Abstract

The effects of microinjections of the excitatory neurotoxin kainic acid (2 mg/ml; 49 ± 1 nl) on the mechanically induced tracheobronchial cough, sneeze, and solitary expulsions from the trachea were examined in 11 anesthetized rabbits. Kainic acid was injected into the medulla (1.6–2.8 mm rostral to the obex, 1.4–1.6 and 2.9–3.2 mm below the dorsal medullary surface). Blood pressure, esophageal pressure (EP), and electromyograms (EMGs) of the diaphragm (DIA) and abdominal muscles (ABD) were recorded. Kainic acid reduced the number of coughs (means ± SE) from 3.8 ± 2.0 to 0.9 ± 0.7 ($p = 0.016$), the amplitude of DIA cough from 90 ± 11 to 42 ± 13 % ($p = 0.004$), ABD EMG moving average from 103 ± 9 to 37 ± 15 % ($p = 0.006$), and inspiratory from 0.67 ± 0.13 to 0.36 ± 0.12 kPa ($p = 0.013$) and expiratory EP from 1.70 ± 0.54 to 0.89 ± 0.46 kPa ($p = 0.008$). Kainic acid had no effect on the number of sneeze reflexes nor did it affect solitary expulsions from the trachea. These effects were accompanied by significant increases in systemic blood pressure and respiratory rate. Spatiotemporal analysis of the cough and sneeze reflexes revealed increases in the duration of cough active expiratory phase, in the intervals between maxima of DIA and ABD EMG discharges, and in the active portion of total cough phase duration. Our findings suggest a diverse role of raphe neurons in the central control

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_6,

of motor airway responses such as coughing and sneezing. A complex function of raphe neurons in the generation of the cough motor pattern also is suggested.

Keywords
Cough motor pattern • Kainic acid • Rabbits • Sneezing • Tracheobronchial cough

1 Introduction

The raphe nuclei are distributed near the midline of the brainstem along its entire rostro-caudal extension. The raphe nuclei are heterogeneous populations of neurons with poorly defined cytoarchitectonic limits which vary in size, shape, and density of cell bodies (Felten and Cummings 1979). These neurones are proposed to transmit and transform sensory information that influences breathing, modulate respiratory drive, and have a stabilizing influence on the respiratory pattern produced by the Bötzinger complex-ventral respiratory group neurons (Böt-VRG) (Smith et al. 2007; Shannon et al. 2004; Lindsey et al. 1994, 2000) There is anatomical evidence of axonal projections of medullary raphe neurons which serve as 'intermediate relays' in the VRG and in the pontine respiratory group (Nuding et al. 2009; Bianchi et al. 1995). Consistent with the role of raphe in the control of reflex behaviors is the fact that kainic acid microinjections into the area of pallidus and obscurus raphe nuclei abolish coughing in anesthetized cats (Jakus et al. 1998). The raphe neurons also participate in the control of expiratory expulsions originating from the trachea in rabbits (Poliacek et al. 2008). In the present study we tested the hypothesis that the elimination of kainate (glutamate) sensitive neurons within the medullary raphe midline in rabbits would reduce the tracheo-bronchial cough reflex, with a possibly smaller effect on the tracheal solitary expulsion or sneezing. We also expected to observe changes in the timing of cough motor pattern.

2 Methods

2.1 General Procedures and Stimulation

All procedures were performed in accordance with the laws, rules, and regulations of Slovakia and EU concerning the animal experiments. The Ethics Committee of Jessenius Faculty of Medicine in Martin, Sloovakia approved the protocols.

The experiments were performed in 11 rabbits (6 chinchilla, 5 New Zealand white; 3.83 ± 0.13 kg) of either sex, anesthetized with sodium pentobarbital (Biowet, Pulawy; 38 mg/kg, i.p.). Supplementary doses of the anesthetic agent were administered (1–3 mg/kg, i.v.) as needed. Atropine (Biotika; 0.15 mg/kg, i.v.) was given at the beginning of the experiment in order to reduce bronchial secretion. The trachea, femoral artery, and vein were cannulated. The animals were allowed to breathe spontaneously a gas mixture of 30–60 % oxygen, balanced with nitrogen. Arterial blood pressure (BP), end-tidal CO_2 concentration ($ETCO_2$), respiratory rate (RR), and body temperature were monitored continuously. Body temperature was maintained at 38.0 ± 0.5 °C. Periodically, samples of arterial blood were used for blood gases and pH analysis.

Bipolar fine wire hook electrodes were placed in the crural diaphragm (DIA) and bilaterally in the transversal abdominal, or the external oblique abdominal muscles (ABD) for electromyogram (EMG) recordings. A soft balloon was inserted into the esophagus for the measurement of intrathoracic pressure changes (esophageal pressure – EP).

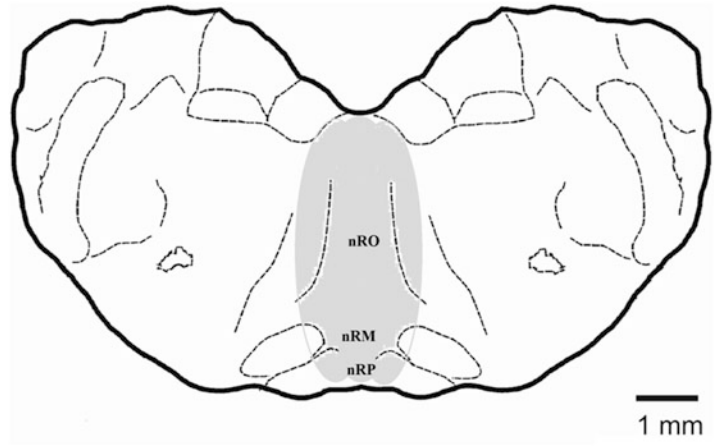

Fig. 6.1 Composite map of brain stem section 100 μm thick showing approximate locations of kainic acid spread after its microinjections (*grey color*). *nRO* nucleus raphe obscurus, *nRM* nucleus raphe magnus, *nRP* nucleus raphe pallidus

Tracheo-bronchial cough was induced by mechanical stimulation of the trachea with a soft nylon fiber or a soft catheter. The fiber was inserted into the trachea and moved back and forth toward the carina four times during one stimulation trial. Sneezing was induced by mechanical stimulation (five touches) of the septum nasi with a soft nylon fiber (Ø 0.25 mm). The cough and sneeze reflexes were defined by a large augmenting burst of DIA EMG activity immediately followed (and partially overlapped) by a burst of expiratory ABD EMG activity (Jakus et al. 1987, 2004), with corresponding inspiratory-expiratory (I-E) oscillations of EP.

Chemical lesions of raphe neurones were induced by microinjections of the excitotoxin kainic acid (Sigma, St. Louis, MO 2.0 mg/ml; 49 ± 1 nl per injection), dissolved in artificial cerebrospinal fluid (aCSF). In the control protocol only aCSF was microinjected (51 ± 1 nl; pH = 7.4). To localize the site of injections, a 4 % solution of Fast Green was also microinjected under pressure using a glass micropipette (tip diameter of 15 ± 2 μm). The micropipette was mounted in a stereotaxic manipulator and micropositioner (David Kopf Instruments; Tujunga, CA) and its tip was inserted into the medulla 1.6–2.8 mm rostral to the obex at the depth of 1.4–1.6 mm and 2.9–3.2 below the dorsal medullary surface, where two microinjections were performed. The ejected volume was determined by direct observation of the solution meniscus in the micropipette through a calibrated grid of the microscope eyepiece. Since kainic acid produces functional inactivation of cell bodies within 30 min (Coyle et al. 1978) we waited 30–40 min before testing the effects. Stimulations of tracheo-bronchial (TB) airways and the nasal mucosa were performed at 1 min intervals; two control TB stimulations were performed before and two after the microinjection, with an interposed nasal stimulation.

The experiment was terminated by an i.v. overdose of pentobarbitone, followed by a saturated solution of KCl. After completing the experiment, the brainstem was removed for histological processing, using the transversal sections of medulla 100 μm thick (Fig. 6.1).

2.2 Data Processing

All EMGs were amplified, filtered (300–3,000 Hz; GRASS), digitalized (12-bit multi-function plug-in ISA card, sampling frequency of 20 kHz), and recorded (WinDaq; Dataq Instruments; Akron, OH) along with the waveforms of BP and EP.

The number of cough efforts being induced during the mechanical probing of the airways (mean number of coughs – CN per four penetrations and touches of carina) and the number of sneeze efforts in response to stimulation of nasal septum (mean number of sneezes – SN per five touches of nasal mucosa) were analyzed in each sequence of trials. The amplitudes of DIA and ABD moving averages as well as of I and E

Table 6.1 Reflex responses after microinjection of 0.2 μg of kainic into the medullary raphe

	TB cough	% dif DIA	% dif ABD	% dif EP I	% dif EP E
Control	3.8 ± 2.0	90 ± 11	103 ± 9	104 ± 25	104 ± 10
Kainic acid	0.9 ± 0.7	42 ± 13	37 ± 15	70 ± 29	39 ± 16
p	p = 0.016	p = 0.004	p = 0.006	p = 0.013	p = 0.008
	SN	% dif DIA	% dif ABD	% dif EP I	% dif EP E
Control	5.0 ± 0.6	188 ± 50	81 ± 9	99 ± 13	92 ± 7
Kainic acid	4.6 ± 0.6	162 ± 40	55 ± 13	87 ± 17	55 ± 7
p	p > 0.05	p > 0.05	p = 0.027	p > 0.05	p = 0.003

TB tracheo-bronchial, *CN* cough number, *SN* sneeze number, *% dif DIA, % dif ABD* peak diaphragm and abdominal EMG activity normalized to control responses, *% dif EP I, % dif EP E* peak inspiratory & expiratory components of esophageal pressure normalized to control responses

parts of EP were normalized to controls – the first sequence of trials. The amplitudes of DIA and ABD EMG moving averages and those of I and E components of the EP recording before and after microinjections were analyzed.

Durations of cough (Wang et al. 2009) related to DIA and ABD activations (TDIA, TABD), augmenting part of DIA (TI = inspiratory cough phase), the time from the maximum of DIA activity to the end of cough related ABD activity (active expiratory cough phase = TE1), the time from the maximum of DIA activity to the end of the cough cycle (expiratory cough phase = TE), and overlapping of DIA and ABD burst (overlap), the time between maxima of DIA and ABD activity (Dif), the quiescent period of the cough cycle (cough E2 phase), the duration of all cough related EMG activity (Tactive), and the whole cough cycle duration (Ttot) were analyzed in each stimulation period. The results are expressed as means ± SE. For statistical analysis a paired *t*-test was applied. The differences of variables were considered significant at $p < 0.05$.

3 Results

Mechanical trachea-bronchial stimulation produced coughing. Stimulation of the nasal septum resulted in vigorous sneezing. Kainic acid microinjections (two injections per penetration) decreased the number of coughs as well as I and E efforts of trachea-bronchial coughs (Table 6.1). We detected a significant decrease in the cough DIA and ABD EMG moving averages and related esophageal pressure amplitudes after kainic acid microinjections in comparison with the control. Kainic acid lowered the amplitude of ABD EMG moving average and the E part of esophageal pressure amplitude during sneezing.

Following the raphe lesions we detected significant increases in the cough active expiratory phase, also in time intervals between maxima of DIA and ABD EMG discharges, and in the active portion of total cough duration (Table 6.2) No changes in 'timing' (temporal features) of sneezing were detected. Neither spatial nor temporal differences were found before and after the raphe lesions in the 'expiration reflex-like' expulsions induced from the trachea. Control microinjections of aCSF had no significant effect on the analyzed reflexes. Kainic acid lesions in the raphe region also were accompanied by significant increases in systemic BP (from 10.76 ± 0.9 to 12.03 ± 0.8 kPa; $p = 0.003$) as well as in RR (from 19.3 ± 2 to 23.9 ± 2 breaths per minute; $p = 0.01$).

4 Discussion

The main finding of our study is a diverse effect of raphe neurons inhibition by kainic acid on the control of coughing and sneezing in the anesthetized rabbit. We also found a significant reduction in the number of tracheo-bronchial coughs and both I and E cough efforts, while only expirations were moderately reduced in case of sneeze. Our study showed that, unlike

Table 6.2 Temporal cough parameters (in ms)

	Control	Kainic acid
Dif	108 ± 11	183 ± 36*
Overlap	176 ± 39	90 ± 43
TABD	332 ± 67	304 ± 65
Tactive	545 ± 63	772 ± 41*
TDIA	391 ± 38	557 ± 45
TE1	249 ± 38	350 ± 51*
TE2	226 ± 129	262 ± 210
TE	475 ± 157	612 ± 190
TI	297 ± 39	422 ± 48
Ttot	772 ± 179	1,034 ± 185

Dif duration between maxima of DIA and ABD, *Overlap* overlapping of DIA and ABD discharge, *TABD* duration of abdominal muscles activity, *Tactive* duration of active cough phase = TI + TE1, *TDIA* duration of diaphragm activity, *TE1, TE2, and TE* duration of active expiratory, quiescent expiratory period, and the whole expiratory phase of cough, respectively, *TI* duration of cough inspiratory phase = elevating part of DIA activity, *Ttot* the whole cough cycle duration

*$p < 0.05$ compared with control

*$p < 0.05$ vs. Control

sneezing, the medullary raphe region rostral to the obex participates in the control of cough pattern timing.

The kainic acid is an excitatory neurotoxin destroying neuronal cell bodies (Coyle et al. 1978); thus interrupting the connections of affected neurons in a neuronal network. Motor patterns of breathing, coughing, and likely of the expiration reflex are generated by a common respiratory/cough central pattern generator located in the Böt-VRG area of the medulla (Smith et al. 2007; Shannon et al. 2004). The nucleus raphe obscurus is interconnected with the pre-Böt complex and with the hypoglossal motor nucleus in the brainstem (Ptak et al. 2009). Neurons of the raphe obscurus and parvus contain a population of serotoninergic and substance P expressing neurons. Both 5-hydroxytryptamine (5-HT) and substance P are released from the 5-HT neurons of the nucleus raphe obscurus under baseline conditions. These neurotransmitters are supposed to act on 5-HT2, 5-HT4, and neurokinin-1 receptors providing tonic excitation of pre-Böt neurons and n. XII motoneurons (Ptak et al. 2009). Peever et al. (2001) have described that in medullary brain slices of the neonatal rat focal microinfusion of kainic acid into the midline raphe cause a disruption of respiratory output, suggesting that this region could be a probable source of endogenous 5-HT that may provide tonic drive to the respiratory network. In addition, the raphe neurons influence quiet breathing and modulate respiratory drive in the cat (Shannon et al. 2004; Lindsey et al. 1994, 2000). We assume that our kainic acid microinjections were capable of affecting a significant number of raphe neurons, possibly resulting in altered respiratory output. In addition, we detected a mild, but significant, increase in RR and a rise of systemic BP after kainic acid microinjections in our animals. Analogos experiments by Poliacek et al. (2008) also showed a higher RR after kainic acid injected into the same area in rabbits; however, no systematic changes in BP were seen. In cats, when microinjections were applied to the raphe midline rostral to the obex, consistent decreases in arterial BP, accompanied by altered respiratory pattern, but with no changes in RR, were found (Jakus et al. 1998). Current models of cardiorespiratory regulation place the medullary raphe neurones within the network that controls the breathing and cardiovascular systems (Jakuš et al. 2004; Lindsey et al. 1994, 1998).

Previous results with kainic acid lesions within the medullary raphe in cats (Jakus et al. 1998) suggest that these neurons provide excitatory input to the neuronal networks responsible for the generation of cough and expiration reflexes. Removal of this facilitatory drive results in the elimination of both reflexes. Our kanic acid microinjections in rabbits decreased all monitored cough parameters and even altered some temporal characteristics of residual post-microinjection coughs. These findings are consistent with altered cough-related drive from the raphe area into the cough central pattern generator. It is unclear if the same, possibly serotoninergic, neuronal pathway that influences breathing also is involved in changes of cough reflex. However, our recent study in cats (Poliacek et al. 2012) showed that the medullary raphe region rostral to the obex participates in the control of cough motor pattern timing by a codeine-sensitive

mechanism. The existence of a 5-HT connection between the raphe nuclei and the pre-Böt complex would seem supportive to the idea of the raphe nuclei having to do with the respiratory pattern generation (Lindsey et al. 1994, 1998; Sessle et al. 1981). The fact that raphe neurons take part in I-E phase switching in rabbits (Wang et al. 2004) and that 5-HT and μ-opioid receptors are involved with signaling pathways in some inspiratory neurones (Manzke et al. 2003) raises the possibility that a single mechanism residing in the raphe area underlies all the aforementioned phenomena.

In the present study we detected a significant decrease in expiratory efforts during sneezing after kainic acid injections into the raphe area, with no alterations in the number of sneezes. The control of abdominal muscles, which are vigorously activated during coughing and sneezing, is coordinated at several levels of the brainstem regions, including the raphe nuclei (Billig et al. 1999). However, a possible contribution of the raphe pathway to expiratory drive during cough and sneeze has not yet been evaluated. Our data show a markedly different modulation of the cough vs. sneeze reflex by the medullary raphe, suggesting a limited contribution of the respiratory/cough central pattern generator (CPG) in generation of the sneeze reflex. This view is consistent with a concept of separate neuronal circuits controlling the expression *vs.* motor pattern of behavior (Bolser et al. 2003) and a concept of overlapping neuronal circuits (e.g., respiratory/cough CPG) being able to reconfigure to meet the need of different motor patterns (Bolser et al. 2006).

Our study shows that chemical lesions in the medullary raphe neurons resulted in diverse effects on the central control of cough and sneeze reflexes in rabbits. The results are consistent with the hypothesis that brain stem neurones may create a multifunction network that controls motor patterns of breathing, coughing, sneezing, and other reflex behaviors (Smith et al. 2007). The raphe nuclei could represent an important part of these control circuits.

Acknowledgements We gratefully acknowledge the technical assistance Iveta Najslova, Ing. Peter Machac, and Mgr. Ivana Šimerová. Supported by VEGA grants 1/0038/09 and 1/0126/12, and co-financed from the European Regional Developmental Fund. In addition, this work also was in part supported by the Slovak Research and Development Agency contract No. APVV-0189-11.

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

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Detection of *Chlamydophila Pneumoniae* Antigens in Patients with Chronic Cough

7

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Abstract

The aim of this study was to analyze the rate of *Chlamydophila pneumoniae* infection in adults with symptoms of chronic cough. The study was conducted in 83 hospitalized patients aged 18–67 suffering of chronic cough. The control group consisted of 20 healthy age-matched subjects without any respiratory symptoms. Bacteriological tests on the presence of *Chlamydophila pneumoniae* antigen were performed in throat swabs by indirect immunofluorescence technique using monoclonal antibodies labeled with fluorescein isothiocyanate. The rate of *Chlamydophila* infected patients was examined in relation to age and gender. The *Chlamydophila pneumoniae* antigen was detected in 15 (18 %) out of the 83 patients; about equally in both genders. Furthermore, we found that the patients aged 28–37 constituted the age group that most frequently tested positive for *Chlamydophila pneumoniae*. Unraveling the presence of *Chlamydia* infection in chronic cough patients enables to introduce a timely implementation of effective therapy and thus can prevent distant complications.

Keywords

Antigen detection • Bronchitis • *Chlamydophila pneumoniae* • Chronic cough • Infection • Pneumonia

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_7,

1 Introduction

Chlamydophila pneumoniae is a Gram-negative obligate intracellular bacterium entirely dependent on energy produced by infected host cells, where it replicates forming characteristic cytoplasmic inclusions. This microorganism can exists in two forms: an infectious form – elementary bodies (EBs) and a metabolic form – reticulate bodies (RBs) (Correia et al. 2005). *Chl. pneumoniae* as all other species of *Chlamydia* have a unique biphasic developmental cycle. The lifecycle has two distinct phases: a nonreplicating extracellular infectious phase and an obligate intracellular non-infectious replicating phase (Campbell 2002). *Chl. pneumoniae* infection is initiated by the attachment of elementary bodies to the cell surface. *Chlamydial* EBs become internalized into the host cell and they remain inside a phagosome which then avoids fusion with lysosomes. EBs transform into metabolically active RBs which start to replicate inside the phagosomal vesicle also called inclusion. In the acute infection, RBs transform to EBs after multiplication and are released from the host cell to infect new cells (Kroll et al. 2005).

Chl. pneumoniae causes a wide variety of respiratory manifestations. The most frequently recognized illnesses associated with the pathogen are pneumonia and bronchitis. Cases of isolated sinusitis, pharyngitis, or otitis have been reported (Jurkiewicz 2008). The general perception is that *Chl. pneumoniae* causes a mild, atypical pneumonia that is often associated with a low degree of temperature, subacute onset, and is frequently associated with pharyngitis and hoarseness. Cough is common but is often non-productive. A mild leukocytosis is usually present in patients who required hospitalization (Hahn et al. 2002). Humans are the only known reservoir for *Chl. pneumoniae* and transmission is from person to person via respiratory secretions, which usually requires a close contact. The incubation time is several weeks (Lui et al. 2009). The list of diseases associated with *Chl. pneumoniae* infection is growing. It is postulated that the microorganism is involved in the development of chronic diseases such as asthma, sarcoidosis, atherosclerosis, Guirlain-Barre syndrome, Reiter's syndrome, and Alzheimer disease (Specjalski and Jassem 2011; Dowell et al. 2001).

The aim of the present study was to analyze the rate of *Chl. pneumoniae* infection in adults with symptoms of chronic cough, a disorder less frequently being associated with the pathogen.

2 Methods

The investigation was performed in accordance with the Declaration of Helsinki for Human Research of the World Medical Association and the study protocol was accepted by a local Ethics Committee of Wroclaw Medical University in Wroclaw, Poland.

The study material consisted of 83 deep pharyngeal swabs obtained from 55 women and 28 men aged 18–67 years hospitalized due to the symptoms of chronic cough. The control group consisted of 20 age- and gender matched healthy subjects without any respiratory symptoms. We employed an indirect immunofluorescence technique (*Chlamydia* Cel PN-IFT kit, Cellabs Pty Ltd, Sydney, Australia) for the detection of *Chl. pneumoniae* antigen in clinical specimens. A monoclonal antibody to *Chl. pneumoniae* binds specifically to the antigen present in fixed specimens and a second FITC- conjugated goat anti-mouse antibody stains the *Chl. pneumoniae* microorganisms. The most common *chlamydial* forms in specimens are EB's. These appear as bright apple-green fluorescent pin-point, smooth-edged disc shaped bodies (approximately 300 nm in diameter) and can be seen against a background of red counterstained cells. RBs may also be observed. These are 2–3 times larger than the EB's and they either fluoresce evenly or pose dark centers with a halo of fluorescence. A positive diagnosis can be made when fixed stained specimens show at least four or more *chlamydial* EB's. A negative diagnosis can be reported when fixed stained smears are free of

chlamydial organism but cells are present. Irregularly shaped fluorescent material that differs in size from *chlamydial* bodies described as fluoresces white, red, or yellow should be considered nonspecific staining.

3 Results

Figure 7.1 presents the results of pharyngeal swabs examination for *Chl. pneumoniae* in 83 patients with chronic cough. *Chl. pneumoniae* antigen was detected in 15/83 patients (18 %). Stratification of the results in relation to gender demonstrates that positive results were found in 10/55 women (18 %) and in 5/28 men (18 %). Concerning the prevalence in relation to patient's age, *Chl. pneumoniae* infection was most frequent in patients aged 28–37 and the least frequent in those aged 48–57 (Fig. 7.2). In the control group, *Chl. pneumoniae* antigen was detected in one asymptomatic woman, which constituted 5 % of all subjects in this group.

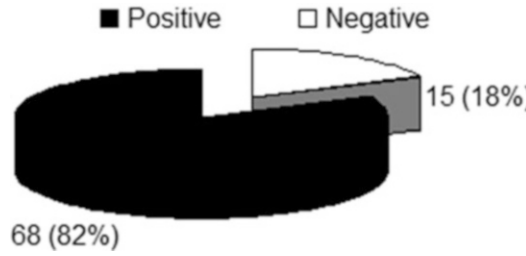

Fig. 7.1 Detection of *Chlamydophila pneumoniae* antigen by indirect immunofluorescence test in patients with chronic cough (n = 83)

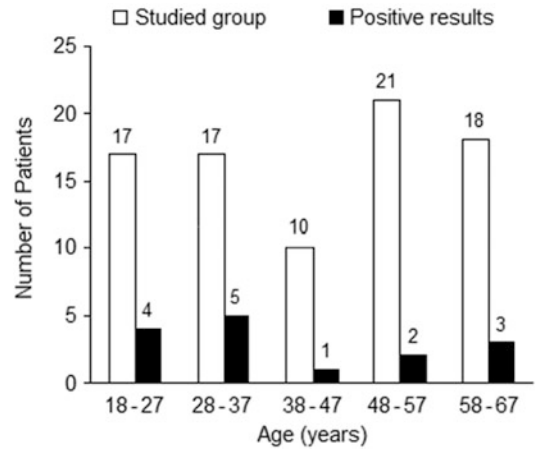

Fig. 7.2 The presence of *Chlamydophila pneumoniae* antigen in patients with chronic cough in different age groups

4 Discussion

Infections caused by *Chl. pneumoniae* are common worldwide. About 50 % of the general population has antibodies against *Chl. pneumoniae*. The only source of infection is another human. It seems that one person in 1,000 suffers from *Chlamydial* pneumonia (Marrie et al. 2012; Burillo and Bouza 2010). Niemhom et al. (2008) used direct immunofluorescence method (DIF) to detect *Ch. pneumoniae* antigen in nasopharyngeal specimens obtained from patients with upper respiratory tract infections and normal individuals. The authors assumed the positive results when both EBs and RBs were visible in specimens and reported the presence of the antigen in 29 of the 37 (78.4 %) persons. IgM and IgG antibodies against *Chl. pneumoniae* by a microimmunofluorescence (MIF) method were determined for the evaluation of the detected *Chl. pneumoniae* and seroconversion. Fifteen samples positive by DIF demonstrated the antibody titers that were interpreted as acute *Chl. pneumoniae* infection, and eight DIF positive samples demonstrated the antibody titers commensurate with chronic infection. Negative results by both DIF and MIF were found in two patients. Concerning healthy subjects, 23 of the 25 were negative by DIF and 20 of the 25 by MIF. The authors suggest that DIF could be an alternative assay for early and accurate diagnosis of acute *Chl. pneumoniae* infection, particularly in conjunction with MIF.

Kaygusuz et al. (2004) used immunofluorescence (IF) method to investigate the antigens of viruses and atypical bacteria in respiratory tract infections (RTI) in pediatric and adult age groups. Sputum (33.6 %) and nasopharyngeal aspirate specimens were obtained from 76 pediatric and 135 adult patients with respiratory tract

infections. *Chl. pneumoniae* antigen detection rates were found to be 17.1 and 13.3 %, respectively. The authors suggest that the IF method could be applied in clinical practice for arriving at a correct diagnosis and for administration of effective treatment.

In the present study, *Chl. pneumoniae* antigen was found, using an indirect immunofluorescence method, in 18 % of patients with chronic cough; the rate of infection notably concerned young and middle-aged adults and was about equal in male and female patients. In the control, healthy adults there were just one case positive for *Chl. pneumoniae* antigen. We conclude that unraveling the presence of *Chlamydia* infection in chronic cough patients enables to introduce a timely implementation of effective therapy and thus can prevent distant complications.

Acknowledgments Supported by Wroclaw Medical University grant 60/Pbmn.

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

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Antioxidant Activity of Herbal Polysaccharides and Cough Reflex

8

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Abstract

The extraction of *Fallopia sachalinensis* leaves resulted in two fractions (FS-1 and FS-2). Chemical and spectral analyses of samples revealed the prevalence of pectic polysaccharides with high galacturonic acid, arabinose, galactose, and rhamnose content. Arabinogalactan with a higher content of phenolic prevailed in the FS-1, whereas rhamnogalacturonan predominated in the FS-2 fraction. Both polysaccharides showed significant antioxidant activity according to DPPH and FRAP assays. Evaluation of antitussive activity in healthy adult conscious guinea pigs after oral application of 50 and 75 mg/kg of the FS-2 polysaccharide extracts showed a significant suppression of cough reflex, without an influence on specific airway resistance. The suppression of cough was comparable with that of codeine.

Keywords

Antioxidant activity • Codeine • Cough reflex • Herbal polysaccharides

1 Introduction

Giant knotweed (*Fallopia or Reynoutria sachalinensis*) is a known invasive plant species native to East Asian, belonging to the Polygonaceae family. Bioactive natural products derived from polygonum species of plants, their structure and mechanisms of action has been already described (El-Hawary et al. 2011). These plants are commonly used in Chinese and Japanese folk medicine (Ogwuru and Adamzenski 2000). The rhizome and root of *Fallopia japonica*, also known by its Chinese name Huzhang, is officially listed in the Chinese Pharmacopoeia (Hu 1993). Huzhang contains resveratrol, polysaccharides, and large amounts of condensed tannins. It can be administrated in the form of an herbal tea, decoction or tincture in therapy of various conditions, including acute microbial infections, viral hepatitis, and goat arthritis (Zhou et al. 2011). The leaves of knotweed contain

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DOI 10.1007/978-94-007-6627-3_8,

anthraquinone glycosides and are be also used in traditional medicines. Contemporary medical use highlights mainly anti-inflammatory effects of its derivates (Shen et al. 2011).

In view of a potential utilization of the *Fallopia sachalinensis* leaves their gross composition has already been investigated (Hromadkova et al. 2010). The content of neutral carbohydrate components of pectic polysaccharides and hemicelluloses was 37.7 %, uronic acid (UA) 10.1 %, and the rest (52.2 %) comprised ash, protein, lignin, and α-cellulose. A sequential extraction of the extractives-free leaves revealed the presence of pectic polysaccharides of the homogalacturonan, rhamnogalacturonan types. In alkaline steps, xylose-rich hemicelluloses were released. Pectin is well known for its bioactive health-promoting properties and use in bio-based products (Silva et al. 2011).

Free radicals may play a pivotal role in the pathogenesis of a number of diseases. The pectic polysaccharides and of an acidic polysaccharide complex from the leaves of *Arctium lappa*, *Aloe barbadensis*, *Althaea officinalis*, *Plantago lanceolata*, *Salvia officinalis*, aerial parts and roots of *Rudbeckia fulgida* were investigated for their ability to inhibit peroxidation of soybean lecithin liposomes by OH radicals (Kardosova and Machova 2006; Capek et al. 2009). Acidic polysaccharides from *Cyclocarya paliuru*, sage, fruit of pumpkin, and from many other medicinal plants exerted significant scavenging effects on DPPH radicals (Capek et al. 2009; Kostalova et al. 2010). We suppose that phenolic components present in the plants participate on their antioxidant activity and antitussive efficacy (Kostalova et al. 2010; Nosalova et al. 2011b). In our previous works, the effects of extracts and structurally defined polysaccharides from medicinal plants on mechanically and chemically induced cough in conscious cats or guinea pigs were tested (Nosalova et al. 2006, 2011a; Sutovska et al. 2007, 2009; Sinha et al. 2011).

The aim of the present paper was to assess antitussive activity of two different polysaccharide fractions extracted from the leaves of *Fallopia sachalinensis* and to compare it with cough suppressive activity of codeine as the most frequently used centrally acting cough suppressive drug in clinical practice (Widdicombe and Ernst 2009; Simera et al. 2010). In addition, we aimed to verify the possible antioxidant activity the polysaccharide extracts.

2 Methods

2.1 Experimental Protocol

The experimental protocol was approved by the institutional Ethics Committee of Jessenius Faculty of Medicine, Comenius University in Martin and complied with Slovakian and European Community regulations for use of laboratory animals. The study was performed in accordance with the revised Declaration of Helsinki of 1983 and followed the criteria of experimental animal's well fare.

Six groups of animals were randomly assigned. Group 1 received vehicle (water for injection) that was given in a dose of 1 ml kg^{-1}; Group 2 was administered codeine (codeine phosphate substance Slovakofarma Hlohovec, Slovakia) in a dose of 10 mg/kg; Groups 3 and 4 were administered pectin polysaccharides FS-1 and FS-2 in a dose 50 mg/kg each; Groups 5 and 6 received pectin polysaccharides FS-1 and FS-2 in a dose 75 mg/kg each. All tested substances (vehicle, codeine, samples of polysaccharides marked as FS-1 and FS-2) were applied orally.

2.2 Plant Material

Leaves of giant knotweed (*Fallopia sachalinensis*) were collected in April 2007 in Česky Krumlov (Czech Republic) and kindly provided by Dr. N. Vrchotová (Institute of Landscape Ecology, AS CR). The leaves were dried at laboratory temperature and ground to a fine powder.

2.3 Chemicals

The Folin-Ciocalteau agent was purchased from Merck (Germany), gallic acid and D-galacturonic acid were from Fluka (Switzerland), the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH*)

and 3-(trimethylsilyl)-propane sulfonic acid sodium salt (TSP) was from Sigma-Aldrich (Germany). All other chemicals used in this study were of analytical grade.

2.4 Antioxidant Activity

The radical scavenging activity (RSA) of the samples was evaluated by the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay (Rao and Muralikrishna 2006; Kostalova et al. 2010). The value EC_{50} was defined as the amount of a sample needed to decrease the initial $^{\bullet}$DPPH concentration to 50 %. The reduction of ferric tripyridyltriazine complex (Fe^{3+}-TPTZ) to ferrous tripyridyltriazine (Fe^{2+}-TPTZ) form by a reductant at low pH was measured according to a slight modification of the ferric reducing antioxidant power (FRAP) assay (Rao and Muralikrishna 2006). The FRAP value was expressed as μmol Fe^{2+}/1 g sample.

2.5 Antitussive Activity

Antitussive activity was investigated as a number of cough efforts and reactivity of the airway smooth muscle *in vivo* in guinea pigs. Adult male guinea pigs, weighing 200–350 g, supplied by Department of Experimental Pharmacology, Slovak Academy of Science, Dobra Voda, Slovakia, were kept in the animal house with food and water *ad libitum* with standard air conditioning system. Animals were kept at least 1 week in quarantine before starting the experiment. Each polysaccharide samples as well as control agents ('positive' codeine and 'negative' vehicle) were tested in individual groups of eight guinea pigs each.

Coughs were recorded in conscious unrestrained guinea pigs, using a double-chamber restricted body plethysmograph box (HSE type 855, Hugo Sachs Elecktronik, Germany); the animal's head protruded into nasal chamber and the neck was sealed with a soft diaphragm. The cough reflex was induced by aerosol of citric acid (concentration 0.3 M) generated by a jet nebulizer (PARI jet nebulizer, Paul Ritzau, Pari-Werk GmbH, Germany; output 51 s^{-1}, particle mass median diameter 1.2 μm) and delivered into the head chamber of the plethysmograph for 3 min. Cough efforts, defined as sudden enhancements of expiratory air flow (approximately × 100-fold) over and above the normal flow. Coughs were recognized by the characteristic animal posture and sounds observed by trained observer and were registered over this time interval. The criteria for cough included a high sound with the mouth open and defined pattern in the sound signal which distinguishes coughs from sneezes (Sutovska et al. 2009; El-Hashim et al. 2011). Measurements of airways function, enhanced pause, and cough were done simultaneously in response to inhaled 0.3 M citric acid, in conscious unrestrained guinea pigs, using whole body plethysmography (HSE type 855, Hugo Sachs Elecktronik, Germany).

The airway smooth muscle reactivity *in vivo* was expressed as specific airway resistance calculated by time difference between pressure changes in both parts of body plethysmograph during normal breathing pattern (Pennock et al. 1979).

Cough efforts as well as airway resistance were registered before administration and subsequently 30, 60, 120, and 300 min after application of the compound. Minimum a 2-h interval between two measurements was set to prevent adaptation of cough receptors as well as general adaptation of guinea pigs to this kind of irritation.

2.6 Statistical Analysis

Student's *t*-test was used for the statistical analysis of the results. Data are represented as means ± SE. A $p < 0.05$ was considered as threshold for statistical significance. Significance levels of $p < 0.05$, $p < 0.01$, and $p < 0.001$ are shown by one, two or three asterisks, respectively.

3 Results and Discussion

3.1 Antioxidant Activity

Both polysaccharide fractions of *Fallopia sachalinensis* were tested for radical scavenging

activity (RSA) using the DPPH assay, which is a convenient method for screening antioxidant compounds. Galacturonic acid exhibits very strong antioxidant activity *in vitro*, as reported earlier (Rao and Muralikrishna 2006; Capek et al. 2009). Therefore, the presence of galacturonic acid might be responsible for the radical scavenging effect (Lin et al. 2009). In our case, fraction FS-1 with lower content of UA (Table 8.1) and higher content of the arabinogalactan showed approximately two times higher antioxidant activity than fraction FS-2 rich in UA. Therefore, these charged molecules cannot be the only reason of this high radical scavenging effect. The phenolic compounds are of special interest due to their known antioxidant properties (Kostalova et al. 2010). The total phenolic (TP) content of FS-1 was significantly higher ($p < 0.01$) than that of FS-2 (Table 8.1). The amount of TP may contribute to the high antioxidative potential. Differences in antioxidant activities can be caused by the presence of a variety of the phenolic compounds. The RSA of samples (DPPH assay) increased significantly in a dose-dependent manner (Fig. 8.1). The result of FRAP assay (expressed as μmol $FeSO_4$ equivalents/1 g sample) showed almost 2.6 times higher activity in FS-1 than in FS-2. Both assays confirmed considerably higher antioxidant activity of sample FS-1 compared with FS-2. The results from *in vitro* experiments demonstrated that the RSA and FRAP values of the samples could be related to their TP content (Table 8.1).

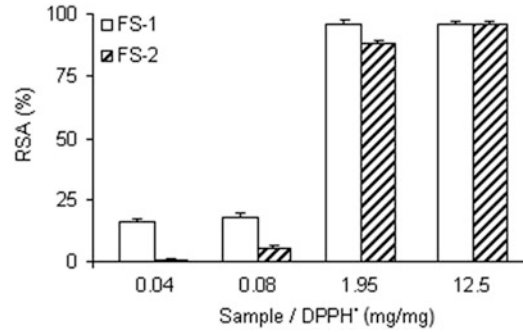

Fig. 8.1 Scavenging abilities of polysaccharide fractions FS-1 and FS-2 extracted from the *Fallopia sachalinensis* leaves against DPPH• radicals. *RSA* radical-scavenging activity, *DPPH* 1,1-diphenyl-2-picrylhydrazyl

3.2 Antitussive Activity

Both polysaccharide fractions of *Fallopia sachalinensis* leaves (sample FS-1 and sample FS-2) were tested for cough-suppressing activity using double-chamber body plethysmography. The antitussive effect was evaluated on the citric-acid induced cough efforts and airways smooth muscle reactivity *in vivo* in adult healthy conscious guinea pigs after oral administration of two different doses 50 and 75 mg/kg of both samples to assess the influence of dose escalation on suppression of cough reflex and specific airway resistance.

Polysaccharide fraction marked as FS-1 showed significant suppression of chemically induced cough efforts already 30 min after oral administration of 50 mg/kg (Fig. 8.2). Cough suppressing activity was more pronounced with the passage of time, and maximal antitussive effect ($p < 0.001$) was achieved at 120 min, but we still observed significant suppression of the number of cough efforts 300 min after FS-1 administration. The greater 75 mg/kg dose of the FS-1 polysaccharide caused slightly increased suppression of the number cough efforts compared with the lower dose (Fig. 8.3). The results of antitussive tests showed that oral administration of FS-2 sample in a dose 50 mg/kg caused significant suppression in the number of chemically induced cough efforts already after 30 min (Fig. 8.2), whereas maximal suppressive effect ($p < 0.001$) after FS-2 was achieved 60 min after its administration. Significant suppression of the number of cough efforts ($p < 0.01$) was observed during the whole experiment. Administration of FS-2 polysaccharide in the larger dose (75 mg/kg) led to a more significant suppression of coughs at 60, 120, and 300 min ($p < 0.001$) compared with the lower dose (Figs. 8.2 and 8.3).

Comparison of cough suppressing activities of both polysaccharides from *Fallopia sachalinensis* revealed a quantitatively higher suppression of the number of cough efforts after application of FS-2 in the higher 75 mg/kg dose. These results led us

Table 8.1 Content of water-soluble polysaccharide fractions FS-1 and FS-2 extracted from the *Fallopia sachalinensis* leaves

					Neutral sugar composition (mol %)					
Sample	UA (%)	TP (%)	FRAP	EC_{50}	Gal	Glc	Man	Ara	Xyl	Rha
FS-1	29.2 ± 3.2	11.9 ± 0.9	674.4 ± 11.8	0.37	28	31	8	24	6	2
FS-2	57.9 ± 5.6	4.1 ± 0.4	253.6 ± 15.2	0.60	29	18	2	32	6	12

Values are means ± SD

UA uronic acid, *TP* total phenolic content, *FRAP* ferric reducing antioxidant power, EC_{50} the lowest mass ratio of an FS sample (mg) to 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) (mg) needed to scavenge 50 % of the initial DPPH, *Gal* Galactose, *Glc* Glucose, *Man* Mannose, *Ara* Arabinose, *Xyl* Xylose, *Rha* Rhamnose

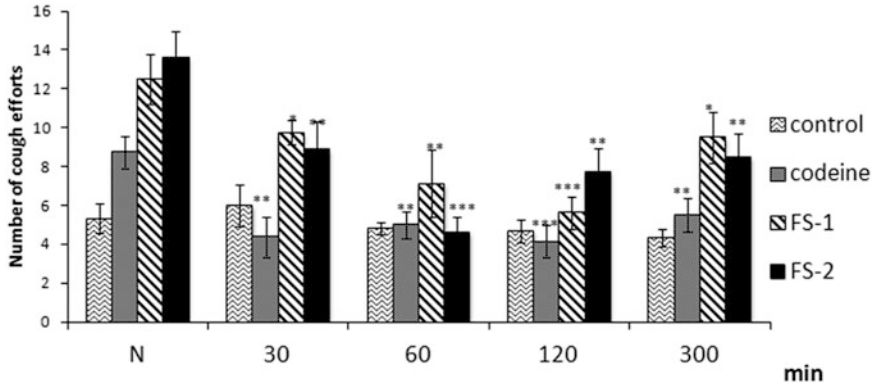

Fig. 8.2 Effects of the fractions FS-1 and FS-2-extracted from the *Fallopia sachalinensis* leaves (both 50 mg/kg) and codeine phosphate (10 mg/kg) on cough reflex. The *first bar* at each time interval represents the control group (water for injection). X-axis represents time intervals of chemical stimulation of airways; y-axis represents the number of coughs. *N* – number of coughs before application of FS-1 and FS-2 samples (Data are means ± SE. *p < 0.05, **p < 0.01, and ***p < 0.001)

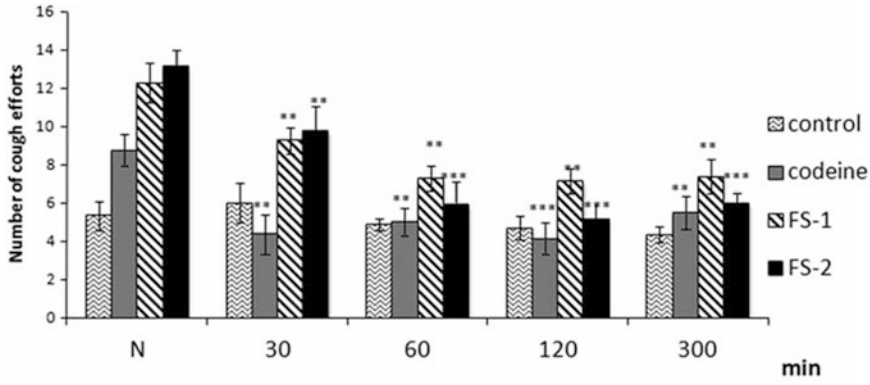

Fig. 8.3 Effects of the fractions FS-1 and FS-2-extracted from the *Fallopia sachalinensis* leaves (both 75 mg/kg) and codeine phosphate (10 mg/kg) on cough reflex. The *first bar* at each time interval represents the control group (water for injection). X-axis represents time intervals of chemical stimulation of airways; y-axis represents the number of coughs. *N* – number of coughs before application of FS-1 and FS-2 samples (Data are means ± SE. *p < 0.05, **p < 0.01, and ***p < 0.001)

to compare the cough suppressive activity of FS-1 and FS-2 in the higher dose and that of codeine – 10 mg/kg; a centrally acting and widely used cough suppressant. We found that sample FS-2 was numerically comparable in suppression of the number of cough efforts with codeine at some time points (Figs. 8.2 and 8.3). Neither did polysaccharide fraction significantly alter airway smooth muscle reactivity, measured as specific airway resistance (sRaw), regardless of the time interval or dose administered. Thus, our results clearly demonstrate antitussive properties of polysaccharides from *F. sacchalinensis* (samples FS-1 and FS-2) in reducing the number of cough efforts induced by citric acid in the guinea pig. These polysaccharides did not provoke any notable adverse reactions.

We tried to address the question of what mechanism could take part in antitussive bioactivity of these molecules. Since the polysaccharides failed to alter airway resistance, bronchodilation is unlikely to underlie their cough suppressive activity. It is dubious that differences in radical scavenging activity are relevant for the difference observed in antitussive activity (higher RSA in sample FS-1, but lower cough suppression). It is possible that different antitussive properties of both polysaccharides are due to their structural differences. A higher molecular weight and a higher content of uronic acid in FS-2 than FS-1 sample could play a role in a more pronounced cough suppressive effect of the former. Earlier studies of antitussive activity of various polysaccharides revealed an increase of antitussive effect with increased content of uronic acid (Nosalova et al. 2011b). Apart from that, polysaccharides, mainly pectins, as present in sample FS-2, with a high molecular size are not completely absorbed after oral administration and stay longer in contact with the mucous terminals of the epipharyngeal nerve. This interaction could decrease cough receptors sensitivity to chemical (citric acid) irritation, and thus indirectly lead to cough suppression (Thirawong et al. 2007; Jakus et al. 2008). It has been described that polysaccharides applied as viscose gel solutions, which prevents irritation of the mucous surface and likely changes cough receptor sensitivity, suppress cough (Nino-Medina et al. 2010). Polysaccharides also increase saliva production, which can contribute to their antitussive effects by activating the swallow reflex possibly competing with cough reflex at the central level.

4 Conclusions

Compositional analysis revealed the dominance of GalA, Ara, Gal, and Rha residues in the water soluble polysaccharides extracted from the knotweed leaves. These polysaccharides are strong free radical scavengers, due likely to both reducing sugars and phenol compounds present in the extracts. The FS-1 and FS-2 polysaccharides, administered orally, demonstrate antitussive activity *in vivo* which is manifest as a reduction in the number of cough efforts induced by citric acid in the guinea pig. Doubling of the dose of polysaccharides from 50 to 75 mg/kg enhanced and prolonged the antitussive effect. The intensity of cough suppression was higher in case of FS-2 than FS-1, and was equipotent to that of codeine; the hitherto archetype of cough suppressants.

Acknowledgements Supported by the Slovak Research and Development Agency (APVV) Grant LPP-0317-09 and Grant 0030-07. This contribution is also a result of the projects 'Center of Experimental and Clinical Respirology II' and 'Support of human resources development using the most modern methods and forms of education' co-financed from the EU sources.

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

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Cytoglobin and Neuroglobin in the Human Brainstem and Carotid Body

9

C. Di Giulio, S. Zara, M. De Colli, R. Ruffini, A. Porzionato, V. Macchi, R. De Caro, and A. Cataldi

Abstract

The aim of the present study was to evaluate the presence of Neuroglobin (Ngb) and Cytoglobin (Cygb) in the solitary tract nucleus (STN) and in the carotid body of human subjects. Transverse serial sections of formalin-fixed, paraffin-embedded brainstems, taken from six subjects, were investigated. Ngb and Cygb are expressed in both the structures. Differences in expression of Ngb and Cygb among dorsal and ventral area of the STN may be related to their different functions and different metabolic demands. Because the STN plays an important role in the processing of cardiovascular and respiratory reflex inputs, Ngb and Cygb may play an integrative central modulatory action for the two systems.

Keywords

Brain stem • Carotid body • Chemoreceptors • Medulla • Neurotransmitter

1 Introduction

The brainstem is involved in many different and complex functions as it contains nuclei which control cardiac and respiratory function, neural networks providing sensory and motor innervations of peripheral structures, and it includes groups of neurons receiving inputs related to the special senses of hearing, vestibular function and taste, or efferent and afferent cerebellar projections. Cardiorespiratory activity is controlled by a network of neurons located within the lower brainstem. The regulatory areas of cardiovascular and respiratory functions are located in the medullary tegmentum, i.e., the area postrema, solitary tract nucleus (STN), and dorsal motor vagal nucleus. The STN contains the afferent chemo- and pulmonary receptors and the dorsal respiratory neurons which switch on during inspiration and relay the activity to the phenic motor nucleus that innervates the diaphragm (Andresen and Kunze 1994). Cardiovascular sympathetic and vagal activities have characteristic

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_9,

discharges that are patterned by respiratory activity. Breathing provides the gas exchange essential for life and is therefore under automatic control, responding to feedback information coming back from many afferents from receptors in the lungs, airway, and from the respiratory chemoreflexes. Respiratory and cardiovascular rhythms are synergistically regulated to ensure adequate ventilation–perfusion matching within the lungs to maintain optimal respiratory gas exchange.

Peripheral arterial chemoreceptors and central respiratory chemoreceptors are crucial for the maintenance of cardiorespiratory homeostasis (Gonzalez et al. 1994). The majority of cardiovascular and respiratory afferent information is transmitted to the STN via the vagus and glossopharyngeal nerves and subsequently processed and integrated by STN neuronal circuits. The STN is located in the dorsomedial medulla and is divided into several distinct subnuclei. One of the two main groups of medullary respiratory neurons is located in the ventrolateral subnucleus of the STN, a region which appears to receive a significant innervation by chemoreceptor afferents. Chemoreceptor reflex input enhances central inspiratory drive and electrophysiological studies indicate that chemo- and baroreceptor afferents are synaptically linked to different populations of STN neurons (Mifflin 1992, 1993; Paton 1998; Silva-Carvalho et al. 1998). It is likely that the neurons excited by both chemo- and baroreceptor inputs exert a synaptic influence on cardiac vagal activity (Spyer and Gourine 2009). These nuclei in the medulla contain many peptides such as endothelin, angiotensin, endorphin, and adrenomedullin (Szilagyi and Ferrario 1981; Sander et al. 1989; Allen et al. 1997). Since carotid body afferents to the STN modulate the respiratory center activity, in this study we analyzed the presence of Neuroglobin (Ngb) and Cytoglobin (Cygb) in the STN and in the carotid body. Cygb and Ngb expression increases in response to various stress stimuli, including hypoxia, and these proteins work as ROS defenders. Ngb is concentrated in neuronal regions that contain mitochondria and its distribution is related to oxygen consumption rates (Pesce et al. 2003). It has been proposed that Ngb enhances O_2 supply to neural components of the STN and may contribute to neuronal survival; the level of Ngb is augmented during ischemia and hypoxia (Sun et al. 2001). Cygb, in turn, would facilitate oxygen diffusion to mitochondria (Burmester et al. 2000) and might function as an oxygen sensor.

2 Methods

2.1 Light Microscopy and Immunohistochemistry

The study was approved by a local Ethics Committee and was performed according to the Italian laws on autopsied human tissues. This study was performed on brainstems sampled during autopsy from six humans. Autopsies were performed within 36 h after death. In all cases, macroscopic and microscopic examination revealed the absence of acute, chronic, localized, or diffuse brain pathology. Brainstems were fixed in 10 % formalin for 7 days. They were cut into 5 mm slices perpendicular to the brainstem axis. Carotid bodies were taken from the right carotid bifurcation, including 20 mm of the common carotid and 20 mm of the internal and external carotid arteries, fixed in 10 % formalin for 1 day. All samples were dehydrated in a series of alcohol solutions of 50, 70, 80, and 95 % (12h in each) and in 100 % alcohol, xylene, and regular paraffin solutions (24h in each). The paraffin-embedded blocks were cut serially and exhaustively into 10 μm thick transverse sections at a calibrated microtome. Final section thickness was measured with a microcator by focusing from the top to bottom surface. The final mean section thickness corresponded to 10 μm. Systematic and uniformly random sampling of the medullary nuclei in their full rostral-caudal extent was performed. In each case, every 80th or 160th section was stained with hematoxylin-eosin and examined for morphometric analysis. The nucleus examined was the STN. The tractus solitarius was always clearly identifiable and we distinguished between dorsal and ventral nuclei. To detect Ngb, Cygb, and Bax protein (pro-apoptotic member of the Bcl-2 protein family), immunohistochemical

analyses were performed by means of Ultravision LP Detection System HRP Polymer & DAB Plus Chromogen (Lab Vision Thermo, CA). Slides were incubated in the presence of mouse monoclonal anti-Ngb (Biovendor, Heidelberg, Germany), anti-Cygb (Abcam, Cambridge, UK), and anti-Bax (Santa Cruz Biotechnology, CA) primary antibodies and then in the presence of specific HRP-conjugated secondary antibodies. Peroxidase was developed using diaminobenzidin chromogen (DAB) and nuclei were hematoxylin counterstained. Negative controls were performed by omitting the primary antibody. Samples were observed by means of light microscopy Leica DM 4000 equipped with a Leica DFC 320 camera (Leica Cambridge Ltd, Cambridge, UK) for computerized images.

2.2 Computerized Morphometric and Image Analysis

After digitizing the images derived from immunohistochemically stained sections, QWin Plus 3.5 software (Leica Cambridge Ltd, Cambridge, UK) was used to evaluate Ngb, Cygb, and Bax expression. Image analysis of protein expression was performed through the quantification of threshold area for immunohistochemical brown color per ten fields of light microscopic observation. Densitometric analysis of immunohistochemistry was determined by direct visual counting of ten fields (mean values) for each of three slides per sample at 40× magnification. QWin Plus 3.5 assessments were logged to Microsoft Excel and processed for standard deviations and histograms. Statistical significance of the results was evaluated using a *t*-test and linear regression test. $p < 0.05$ was taken as indicative of significant differences.

3 Results

We found that both Ngb and Cygb were expressed in the STN and the carotid body glomus cells. The expression of Ngb is higher appreciably in the STN (9.0 ± 0.9 %) than that in the carotid body (2.5 ± 0.2 %; $p < 0.01$) (Fig. 9.1). As regards comparison between dorsal and ventral STN structures, there are no significant differences in the total Ngb amount.

Cygb expression do not show significant variations between the medulla and carotid body samples (0.6 ± 0.05 %) (Fig. 9.2). As regards comparison between the dorsal and ventral STN, there are no significant differences in the total Cygb amount.

Finally, expression of the Bax pro-apoptotic factor is significantly less in the carotid body compared to the STN; (0.22 ± 0.05 vs. 0.44 ± 0.2 %, respectively, $p < 0.01$) (Fig. 9.3).

4 Discussion

The STN is essential for respiratory modulation as it receives multiple visceral afferents, notably having to do with cardiovascular and respiratory functions. One of the most important inputs to the STN is neural discharge emanating from the carotid body; an organ that encompasses oxygen sensitive cells which generate electric signals in response to a decrease in arterial oxygen pressure. These signals are used by the central nervous system to regulate the function of respiratory muscles. The arterial chemoreceptors are thus the gates that carry information concerning the status of systemic oxygen and in effect trigger the hypo- or hyperventilatory response (Gonzalez et al. 1994).

In the present study we found that Cygb is expressed in the human medullary tissue and this protein seems to work in the STN as a 'neuronal globin protein', providing information on O_2 alterations to the respiratory medullary network. In addition, Cygb might also act as a sensor to detect cellular O_2 concentration, with an affinity comparable to the myoglobin P_{50} of 1–2 Torr; (Burmester and Hankeln 2004). Hypoxia upregulates Cygb expression in neurons, suggesting that Cygb could protect neurons against hypoxic damage. Cygb might also be involved in the detoxification of nitric oxide and other reactive oxygen species generated under hypoxic conditions. Thus, the presence of

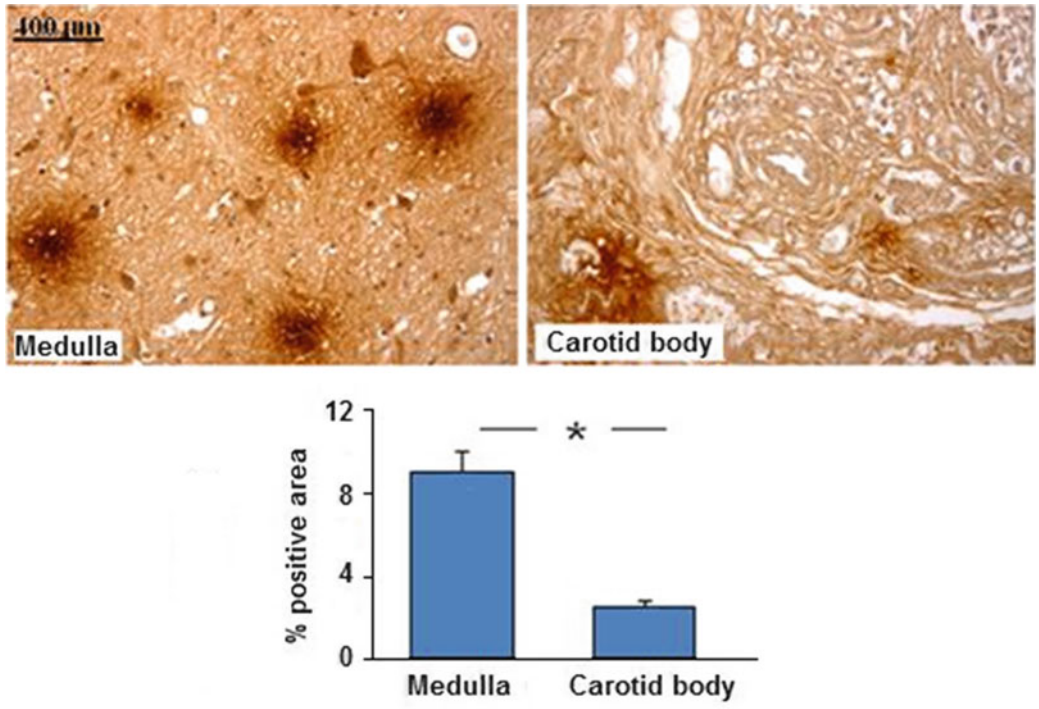

Fig. 9.1 Immunohistochemical (*upper pictures*) and densitometric (*lower bars*) analysis of Ngb expression in the medulla and carotid body (*CB*). Magnification: 20×; *Medulla Ngb vs. CB Ngb, $p < 0.01$

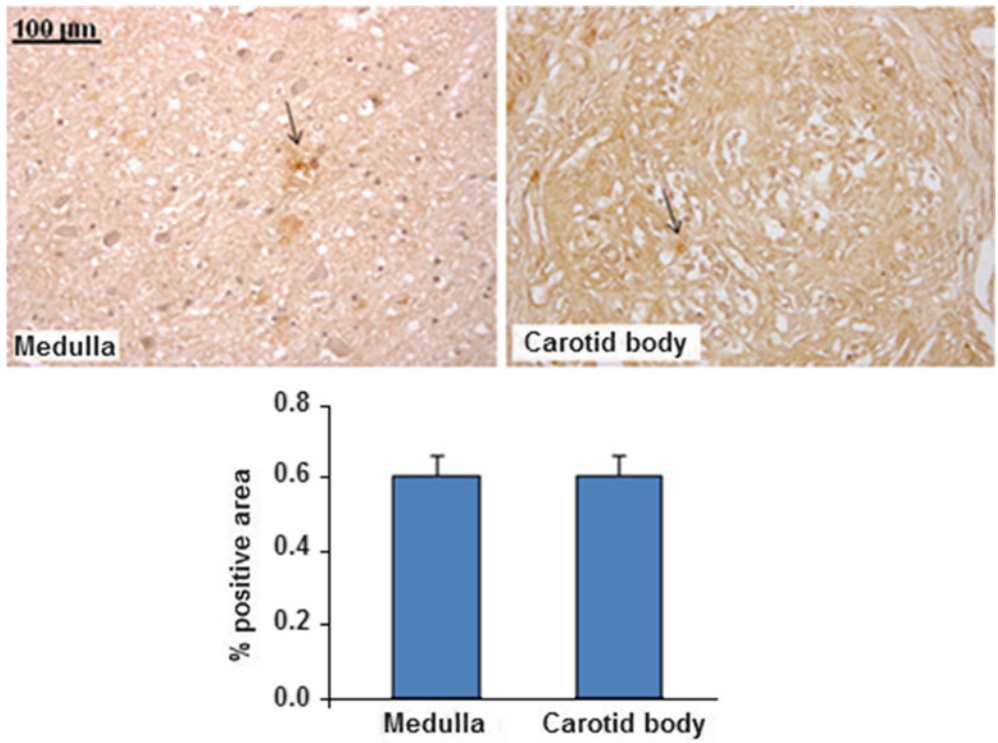

Fig. 9.2 Immunohistochemical (*upper picture*) and densitometric (*lower bars*) analysis of Cygb expression in the medulla and carotid body (*CB*). Magnification 20×; *Arrows* indicate positive staining

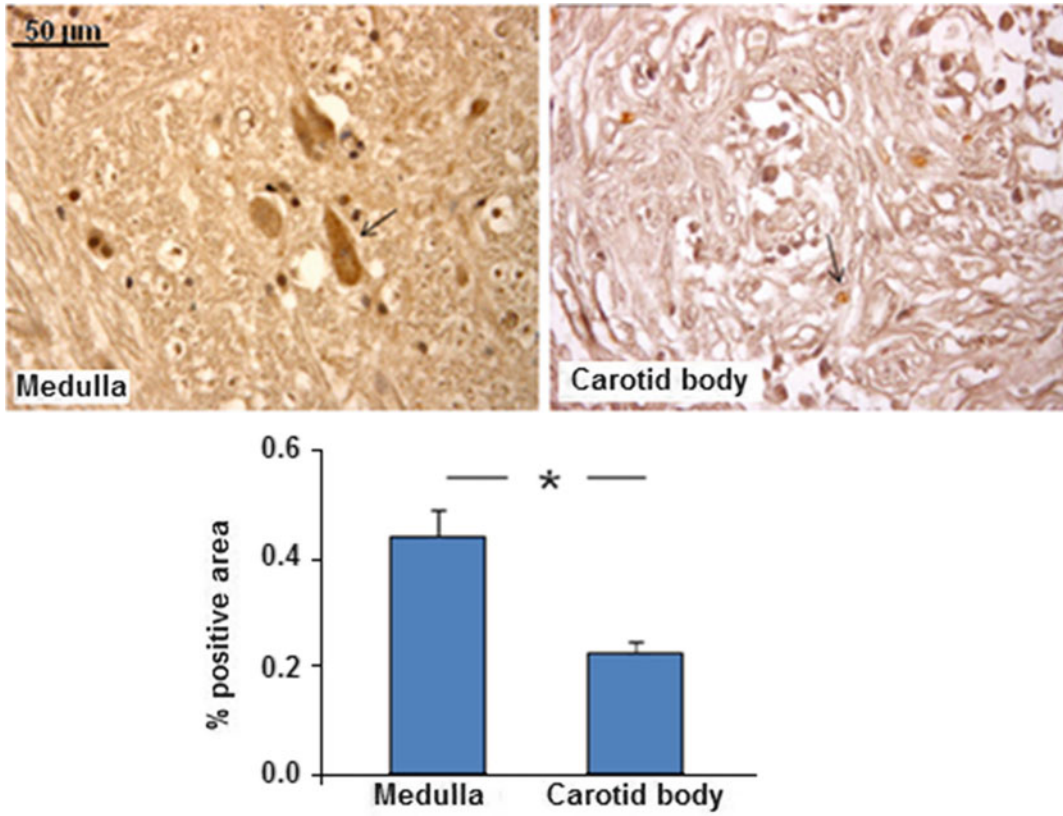

Fig. 9.3 Immunohistochemical (*upper pictures*) and densitometric (*lower bars*) analysis of Bax protein expression in the medulla and carotid body (*CB*) Magnification: 40×; *Medulla Bax vs, CB Bax, $p < 0.01$. *Arrows* indicate positive cells

Cygb as 'a respiratory protein' in the medullary respiratory region may play a role in NO and ROS detoxification. It is known that hypoxia stimulates hypoxia-inducible factor-1 (HIF-1), nitric oxide synthase, and tyrosine hydroxylase production in the medulla (Lahiri et al. 2002; Di Giulio et al. 2003). These molecules might exert neuroprotective effects through diverse mechanisms and their hypoxic-responsiveness depends on O_2-binding proteins, such as Cygb (Bunn and Poyton 1996; Dewilde et al. 2001), which can sense hypoxia and trigger appropriate cell adaptive responses (Burmester and Hankeln 2004). A link between Cygb and HIF is plausible, with Cygb having a protective role for glomus cell plasticity during development. Moreover, we found increased expression of the pro-apoptotic Bax protein in the medulla compared with that in the carotid body. Ngb, a 151-amino-acid protein with a predicted molecular mass of 17 kDa, has recently been identified as a member of the vertebrate globin family (Burmester and Hankeln 2004; Mammen et al. 2002). It is predominantly expressed in nerve cells, particularly in the brain and in the retina (Burmester et al. 2000; Zhu et al. 2002), but it is also present in other tissues (Burmester and Hankeln 2004). The physiological role of Ngb is not well understood, but it has been proposed that it participates in several processes such as oxygen transport, oxygen storage, and nitric oxide detoxification (Burmester and Hankeln 2004). Ngb, as well as Cygb and hemoglobin, may act as a respiratory protein which reversibly binds gaseous ligands (NO and O_2) by means of an Fe-containing porphyrin ring. Further studies are needed to establish the role of Cygb and Ngb in the medulla and to verify

whether the oxygen-sensitive mechanisms are related to the Cygb level in the respiratory neuronal network.

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

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Flow Cytometry in the Diagnosis of Influenza

10

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Abstract

Flow cytometry is used in the analysis of the multi-parameter optical properties of individual particles such as eukaryotic cells, prokaryotic cells, and viruses in the flow system. Virions, or complexes consisting of virus particles attached to the specific antibody in suspension are individually arranged in a linear stream, which flows through the detection device. The parameters measured by the flow cytometer include the volume of the particles or cells, the morphological complexity, the presence of pigments, RNA content, virion surface markers, and enzymatic activity. It is possible to collect two morphological parameters and one or more signals of the fluorescence of a single particle. Multi-parameter analysis provides for the definition a population of cells based on their phenotype. Flow cytometry is characterized by the automatic determination of the value of the parameter set for a large number of individual particles or cells in the course of each measurement. For example, 100,000 or more particles such as virus, bacteria, or fungal spores are analyzed one after another typically over a period of 1 min. The limit of detection in such studies is 100 fluorescing particles per cell. Theoretically, in the case of the influenza virus, this will be one copy of the virion

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_10,

combined in a complex with specific antibodies and with a built-in fluorescent label.

Keywords
Antibody flow cytometry • Fluorescent markers • Influenza virus • Viron

1 Introduction

The research value of flow cytometry can be attributed to the rapid availability of the results of qualitative and quantitative, multi-parameter measurements of cells infected with influenza virus or influenza virions themselves in sufficiently large quantities required for the appropriate statistical calculations. Due to the close relationship of the virus and the host cell the use of this technique makes possible the extensive study of virus-cell interactions including an examination of the process of apoptosis induced by viruses, the expression of cell antigens in response to viral infection, the identification of receptors for the virus on the cell surface and the detection of virus antigen for instance, on the surface or within infected, sensitive cells (Kraj 1991). The present article presents the use of modern methods for the detection of influenza viruses using flow cytometry.

2 Operating Principle of the Flow Cytometer

Flow cytometry is a method for measurement of subpopulations of cells using a device called a flow cytometer (Kawiak and Hoser 1993). With this method, it is possible to evaluate the morphological characteristics of the cells or virus under investigation and the amount of fluorescent dye taken up by their organelles (Baran 1996; Kawiak and Hoser 1993). In this device, individual particles within a liquid stream flow through a beam of light and a detector (Robinson et al. 1997). Depending on the method of staining, it is possible to analyze live or dead cells, viral particles and, if the cytometer is equipped with a separation sorter, homogeneous groups in terms of the selected features in the test cell population or in formed particles. The modern flow cytometer is a device that combines the latest developments from a variety of quite distant disciplines. The principle of operation of the apparatus is similar to that of a fluorescence microscope, but one equipped with a measuring system. The difference lies in the fact that, looking through the microscope the cells or virions are attached to a base such as a glass slide, however, in flow cytometry the cells or virions are analyzed in a flowing liquid stream. Such an arrangement is very convenient for the quantitative measurement of a number of particles, which in turn move to a point at which a number of parameters are measured for each of them. Lasers with fixed or variable power provide illumination; lenses and multilayer filters isolate the desired wavelength of fluorescence emission, which is picked up and amplified by very sensitive photomultipliers. A hydrodynamic system enables the shaping of the stream containing the cells under investigation, so as to place them one after another centrally within the liquid stream. An important condition for a good reading is an appropriate concentration of cells in the sample giving an optimum cellular flow rate. The whole device is controlled by a computer equipped with a system for collecting and storing data, graphical image analysis programs, and software for statistical calculations.

3 Fluorescent Dyes Used in Virological Studies

Fluorescent dyes in flow cytometry are treated as basic reagents through which accurate

information can be obtained concerning the structure, function, and changes in cells infected with influenza virus or in virus alone. As a result of absorption of radiation the electrons of dye atoms are 'punched' to a higher orbital, and then return to their orbital base. Electrons emit the absorbed energy as fluorescence and reflected heat. The energy loss in the form of heat causes the so called Stokes shift that is the displacement of the spectrum of light emitted in the direction of longer wavelengths. All good fluorescent markers used in the diagnosis of influenza should have the following characteristics:

- high capacity for absorption of excited radiation;
- high value of emitted radiation by particles of dye coupled with the target component of the cells or virus under analysis;
- optimal radiation emission wavelength, which would not overlap with any auto fluorescence of the objects under investigations;
- high stability, the fluorescent marker should persist for 10–100 thousand excitations before degradation;
- shortest possible duration (about 1 ns) of the excited state. The shorter this period, the more cycles of excitation/emission occur during the passage through the cytometer detector (Robinson et al. 1997)

Several dyes are available for the staining of the individual components of influenza virus-infected cells or virions. They differ in terms of attachment position to the cellular element under examination, staining of the virus particle, the absorption and emission wavelength, the capacity of live and dead cells to take up stain. Selection of the appropriate fluorescent marker depends on the type of laser used and on exactly what is to be examined.

4 Analysis of Cytometric Measurements

The results of cytometric measurements are presented as graphs called cytograms. The simplest of these is the histogram with fluorescence intensity plotted on the x-axis, and the number of analyzed particles – cells or virions on the Y-axis (Fig. 10.1a). In two-dimensional cytograms (dot plot, density plot, and contour plot) two selected parameters are plotted on the X and Y axes and the particles are presented in the form of dots or contours (Figs. 10.1b, c). After the addition of a Z-axis it is possible to create spatial cytograms, simultaneously showing two features and the size of the particles studied (Fig.10.1d). The use of software enables access to complete statistical information for any given region of the graph. The cytometer can be used to read fluorescence intensity or the degree of laser light scattering, however it is not possible, for instance, to obtain direct information on the size or structure of the surface of for example the influenza virus. These parameters can only be determined from the cytograms indirectly by comparison of the values with standard readings. These include for example, cells with known shapes and sizes, or plastic particles (beads) of known and identical diameter and which have the same constant fluorescence (Darzynkiewicz et al. 1994).

5 Cytometric Studies of Influenza Virus

Influenza continues to be a serious medical problem in the twenty-first century. Cases are recorded in all latitudes and in every age group from infants to the elderly. Members of the *Orthomyxoviridae* family are the cause of this infection. The classification of influenza virus into three types A, B, and C is based on antigenic differences in the structural proteins, which are nucleoprotein and protein matrix. Influenza virus type A can occur in humans and in birds, pigs, horses, and marine mammals. Due to the significant differences in the surface glycoproteins, i.e., hemagglutinin and neuraminidase, 16 subtypes of hemagglutinin (H1-H16) and 9 neuraminidase subtypes (N1-N9) can be identified in type A influenza virus. To date, subtype A (H1N1), A (H1N1) pdm09, A (H2N2), A (H3N2), A (H1N2), A (H5N1) HPAI, A (H9N2), and A (H7N7) HPAI have been isolated in humans. Furthermore, influenza B virus has been found

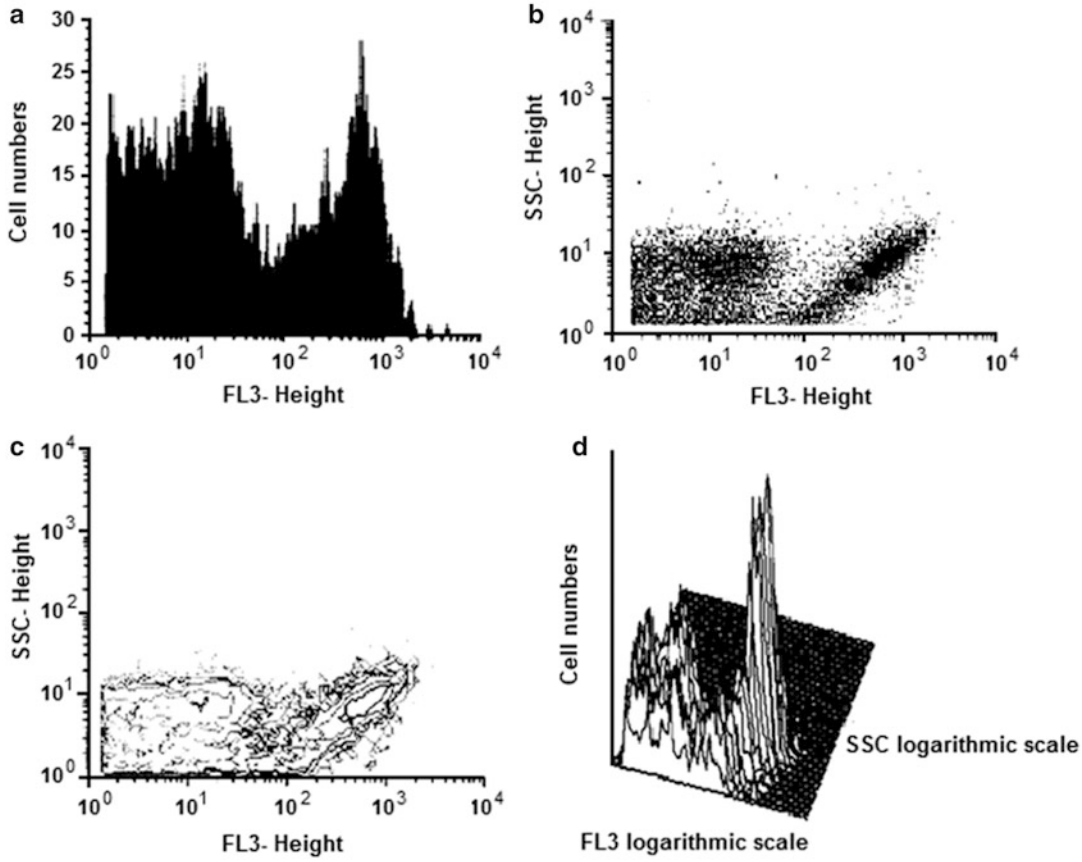

Fig. 10.1 Examples of different cytometric presentations of the same eukaryotic cell suspension. (**a**) Histogram of propidium iodide FL3 fluorescence. (**b**) Two-dimensional dot graph of the cells investigated. (**c**) Two-dimensional contour graph. (**d**) Three-dimensional FL3 logarithmic graph

to occur solely in humans, and type C virus has the ability to infect humans and pigs (Brydak 2008, 2012).

Until relatively recently, the method of flow cytometry was used mainly in hematology and then it was introduced for virological analysis at the end of the last century. Currently, this method is applied successfully in virology and is a valuable tool in the study of the influence of various external factors such as temperature, culture medium, or the quality of the cell lines used in the process of viral replication (Schulze-Horsel et al. 2008). This is very important for the production of influenza vaccines. Monitoring viral titers and the status of cell lines during viral culture is important for the ongoing process and its optimization. Several cell lines have been characterized that have a specific use in the industrial production of the influenza virus which include the Vero cell line (African monkey kidney), the PER.C6 cell line (obtained from the ocular tissue of a child), and the MDCK line (Madin Darby Canine Kidney). Schulze-Horsel et al. (2008) showed that the fluorescein-labeled monoclonal antibodies against the viral protein matrix of influenza A and nucleoprotein make it possible to detect a high accumulation of the protein in adherent MDCK cell line susceptible to infection by this type of virus. This relationship can be detected using flow cytometry already 2–4 h after infection has occurred. However, a significant increase in the titer of influenza virus detected by the classical method is noticeable after a period 4–6 h or later (Schulze-Horsel et al. 2008). Cytometric analysis may also be used for characterization studies on

the repeatability of vaccine production. In addition, the study of cellular processes that occur in cells infected with influenza virus is of great importance for the optimization of the vaccine manufacturing process. Hence, monitoring the production of influenza virus and the dissemination of infection at the cellular level with flow cytometry may provide scientists with vital information about the virus and its impact on infected cells.

Flow cytometry is also used in large-scale studies on the evaluation of the sensitivity of influenza virus to antiviral drugs such as neuraminidase inhibitors, for example, oseltamivir or zanamivir. When compared with other respiratory viruses, it is only in the case of influenza virus infection that treatment is available. In order to ensure effective treatment laboratory tests to detect the presence of influenza virus should be performed without delay. These tests should provide for a quick and accurate diagnosis, which would avoid the use of long-term and often debilitating antiviral therapy. McSharry et al. (2004) reported that a sufficiently high concentration of the anti-viral drug is associated with a reduction in the number of cells infected with influenza virus and this may be analyzed using the appropriate cytometric fluorescent marker coupled with specific antibodies. The researchers draw attention to the much faster availability of results and to a less time-consuming method. However, they state that the cytometric method requires an experienced analyst

6 Conclusions

Previous attempts to use flow cytometry in clinical and virological studies, especially for influenza virus, were relatively few, as evidenced by a small number of scientific reports on this subject. The main limitation of the method of cytometric measurement of influenza virus probably resulted from its very small size. To-date, the following viruses were studied with positive effect: African swine fever virus (ASFV), hepatitis C virus (HCV), HCMV, HIV, and influenza virus. Furthermore, flow cytometry studies were performed on the cell cycle of yeast of the genus *Saccharomyces*, bacteria of the genus *Enterobacteriaceaea* and *Mycobacterium tuberculosis* in which the content of DNA and specific proteins was measured (Wozniak-Kosek et al. 2003; McSharry 1994; Darzynkiewicz et al. 1994). Similar studies are theoretically possible in protozoa and algae. With technical advances, flow cytometry has also become increasingly common in virological studies especially in clinical virology, where this technique can replace or supplement the very time-consuming methods for detection and identification of influenza virus and the methods for assessing the susceptibility of these viruses to antiviral drugs. The flow cytometer can also be helpful in studies concerned with the construction of seasonal influenza vaccine composition. To-date, the laboratory diagnosis of influenza infection has been based on the demonstration of the presence of the virus or its nucleic acid and identification of virus in clinical specimens. Furthermore, analysis includes the assessment of the body's immune response to developing infections, i.e., the detection of antibody (Brydak 2008). Standard methods of influenza virus culture are mainly used for diagnosis. However, their sensitivity is significantly lower than that of molecular methods (Jagus et al. 2010; Ghebrememedhin et al. 2009). It is worth noting that in this particular case, confirmation of etiology by culture is only achieved after 3 days. However, according to current WHO recommendations targeted influenza therapy should be started within 36 h of the first symptoms of infection (Jagus et al. 2010; WHO report 2005). Hence, the importance of seeking innovative fast methods of identification, not only of the influenza virus, since the time required to perform tests utilizing molecular techniques is approximately 5–8 h, while flow cytometry takes approximately 1 h for the analysis of thousands of formed particles (infected cells in cell cultures or of virions).

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

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Accuracy of Rapid Influenza Detection Test in Diagnosis of Influenza *A* and *B* Viruses in Children Less Than 59 Months Old

11

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Abstract

Influenza burden among children is underestimated. Rapid influenza diagnostic tests (RIDTs) may be helpful in the early diagnosis of the disease, but their results should be interpreted cautiously. The aim of our study was to estimate the accuracy of the rapid influenza detection test BD Directigen™ EZ Flu A+B (Becton, Dickinson and Company, Sparks, MD) used among children with influenza-like illness (ILI) consulted in the ambulatory care clinics. A total number of 150 patients were enrolled into the study. The inclusion criteria were: age of the child less than 59 months, presentation of ILI according to CDC definition (fever >37.8 °C, cough, and/or sore throat in the absence of another known cause of illness), and duration of symptoms shorter than 96 h. In all patients two nasal and one pharyngeal swab were obtained and tested by RIDT, RT-PCR, and real time RT-PCR. For or influenza A(H1N1)pdm09, virus sensitivity of RIDT was 62.2 % (95 %CI 53.4–66.5 %), specificity 97.1 % (95 %CI 93.4–99 %), positive predictive value (PPV) 90.3 % (95 %CI 77.5–96.5 %), and negative predictive value (NPV) 85.7 % (95 % CI 82.4–87.3 %). For influenza B, virus sensitivity was 36.8 % (95 %CI 23.3–41.1 %), specificity 99.2 % (95 %CI 97.3–99.9 %), PPV 87.5 % (95 %CI 55.4–97.7 %), and NPV 91.5 % (95 % CI 89.7–92.1 %). We conclude that the RIDT immunoassay is a specific, but moderately

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_11,

sensitive, method in the diagnosis of influenza type A and is of low sensitivity in the diagnosis of influenza B infections in infants and children.

Keywords
Children • Diagnosis • Influenza • Pharyngeal swabs • Rapid influenza diagnostic test • Virus

1 Introduction

Influenza is an acute viral disease which burden among children is underestimated (Principi et al. 2004). Depending on age, incidence rates of influenza may be 1.5–3.0 times higher than those for adults and are estimated to be between 10 and 40 % each year (Long et al. 1997). Influenza in the pediatric population leads to a significant increase in primary care visits, emergency department visits and hospitalizations due to complications (Neuzil et al. 2000).

The diagnosis of influenza is based on clinical symptoms and results of additional laboratory tests confirming presumptive diagnosis, including rapid influenza detection tests (RIDTs), real time polymerase chain reaction (RT-PCR), direct immunofluorescence assay (DIA), or viral culture. There are no typical symptoms for influenza, but the disease may be suspected in the presence of: the acute onset, cough, and fever >38.5 °C (Landry 2011; Friedman and Attia 2004). The presumptive diagnosis requires confirmation and the viral culture has been considered the gold standard for influenza diagnosis, but the delay in obtaining results makes it impractical for clinical-decision making. The polymerase chain reaction is more sensitive than standard viral culture, but is not widely available and expensive (Landry 2011). As an alternative, RIDTs are relatively inexpensive and can provide timely information for clinical diagnosis (Uyeki 2003). The positive aspects of a timely diagnosis of influenza include the opportunity to provide antiviral therapy, allowing implementation of measures to limit the spreading of the disease, avoiding unnecessary antibiotic therapy and ambulatory and hospital testing and costs (Angoulvant et al. 2011; Jennings et al. 2009; Esposito et al. 2003). However, physicians must be aware that the accuracy of interpretation of rapid-test results depends on many factors including: clinical presentation of the disease, duration of symptoms, age of the patient, sample type, prevalence of influenza in the community, test characteristics, even previous vaccination against influenza using life-attenuated vaccine (Poehling et al. 2011).

The aim of our study was to estimate the accuracy of the rapid influenza detection test BD Directigen™ EZ Flu A+B used among children younger than 59 months with symptoms of influenza-like illness (ILI) consulted in the ambulatory care clinics.

2 Methods

The children in whom the accuracy of the BD Directigen™ EZ Flu A+B test was assessed also were used as subjects in the accompanying paper in which we described the clinical outcomes of influenza caused by viruses type A and B (Nitsch-Osuch et al. 2013). The assessment of the rapid influenza test efficacy, perceived as a single unrelated ramification of the study and a disconnected technical and research issue, was analyzed separately and presented herein. The study protocol was approved by a Local Ethics Committee and informed consent was obtained from the children's parents.

The study was conducted in the double autumn and winter seasons from 2009 to 2011.

A total of 150 children (47 % boys and 53 % girls) younger than 5 years (72 % children older than 24 months, 5 % younger than 12 months, 23 % children aged 12–24 months) were enrolled into the study. All patients were consulted at the ambulatory care clinics in Warsaw, Poland by general practitioners or pediatricians and presented symptoms of influenza-like illness shorter than 4 days. In 50 % of the patients, symptoms lasted longer than 48 h, in 9 % shorter than 24 h, in 41 % between 24 and 48 h. Fever >37.8 °C was reported in all patients, cough and/or sore throat was present in 93 % cases.

Biological material (nasal and pharyngeal swabs) taken from the patients were tested with a RIDT (BD Directigen™ EZ Flu A+B; Becton, Dickinson & Company, Sparks, MD). The isolation of viral RNA was conducted with Maxwell® 16 Viral Total Nucleic Acid Purification Kit (Promesa Corp., USA) and one-step RT-PCR was carried out with a Transcriptor RT-PCR Kit (Roche Diagnostics, Switzerland). Samples positive for influenza type A virus in RT-PCR were tested with a real time RT-PCR (Invitrogen SuperScript™ III Platinum® One-Step Quantitative RT-PCR System; Invitrogen, USA).

The Directigen EZ Flu A+B test was described by manufacturer as a chromatographic assay to qualitatively detect influenza A and B viral antigens in samples processed from respiratory specimens. When specimens were processed and added to the test device, influenza A or B viral antigens bound to anti-influenza conjugated antibodies to visualize particles in the corresponding A and B test strips. A positive result for influenza A was visualized as a reddish purple line at the test 'T' and control 'C' positions in the Directigen EZ Flu A read window. A positive result for influenza B was visualized as a reddish purple line at the test 'T' and control 'C' positions in the Directigen EZ Flu B read window.

Sensitivity, specificity, positive and negative predictive values (PPV and NPV), positive and negative likelihood ratio (LH+ and LH−), and kappa score of RIDT compared to RT-PCR were separately calculated for influenza type A and influenza type B virus. 95 % confidence intervals were calculated for all values. The statistical analyses were performed using SPP (ver. 15.0 for Windows, Chicago, IL).

3 Results

According to RT-PCR results the total number of 64 cases of influenza was diagnosed (incidence rate 40 %): 19 cases of influenza caused by virus type B and 45 cases of influenza caused by type A virus. *Real time* – RT-PCR revealed that all cases of influenza A were caused by subtype A(H1N1)pdm09. The accuracy of the RIDT was calculated for influenza type A (H1N1)pdm09, influenza type B. For influenza type A(H1N1)pdm09 infection the following results were obtained: 28 true positive, 102 true negative, 3 false positive, and 17 false negative. For influenza type B infection there were obtained: 7 true positive, 130 true negative, 1 false positive, and 12 false negative results. Values of sensitivity, specificity, PPV, NPV, LH+, LH− and kappa score of the rapid influenza detection test BD Directigen™ EZ Flu A+B compared to RT-PCR are presented in the Tables 11.1 and 11.2.

Table 11.1 Accuracy of rapid influenza detection test BD Directigen™ EZ Flu A+B compared to RT-PCR results for influenza A(H1N1)pdm09 virus

	Value	95 % CI
Sensitivity	62.2 %	53.4–66.5 %
Specificity	97.1 %	93.4–99 %
PPV	90.3 %	77.5–96.5 %
NPV	85.7 %	82.4–87.3 %
LH+	21.8	8.0–64.9
LH−	0.39	0.34–0.50
Kappa score	0.65	0.51–0.72

PPV positive predictive value, *NPV* negative predictive value, *LH+* positive likelihood ratio, *LH−* negative likelihood ratio

Table 11.2 Accuracy of rapid influenza detection test BD Directigen™ EZ Flu A+B compared to RT-PCR results for influenza B virus

	Value	95 % CI
Sensitivity	36.8 %	23.3–41.1 %
Specificity	99.2 %	97.3–99.9 %
PPV	87.5 %	55.4–97.7 %
NPV	91.5 %	89.7–92.1 %
LH+	48.3	8.6–296.9
LH−	0.66	0.59–0.79
Kappa score	0.48	0.27–0.55

PPV positive predictive value, *NPV* negative predictive value, *LH+* positive likelihood ratio, *LH−* negative likelihood ratio

4 Discussion

The present study shows that rapid influenza detection test BD Directigen™ EZ Flu A+B has moderate sensitivity (62.2 %) in detection of influenza A(H1N1)pdm09 virus and low sensitivity in detection of influenza B infection (36.8 %) among children with influenza-like illness younger than 59 months consulted in ambulatory care settings. The specificity of the test was high for both influenza A(H1N1)pdm09 virus (97.1 %) and influenza type B virus (99.2 %). Positive predictive values were also high and ranged from 87.5 % for influenza type B virus to 90.3 % for influenza type A(H1N1) pdm09 virus. Negative predictive values ranged from 85.7 % for influenza A(H1N1)pdm09 virus to 91.5 % for influenza B.

The values describing the accuracy of the rapid influenza detection test BD Directigen™ EZ Flu A+B calculated in our study were lower than those indicated by the manufacturer and calculated for viral culture as a gold standard. Our results concerning the accuracy of BD Directigen™ EZ Flu A+B test for detection of influenza A(H1N1)pdm09 are in agreement with the studies conducted by Karre et al. (2010) who found the sensitivity of this test of 48.7 %, specificity of 96.5 %, PPV of 88.6, and NPV of 77.3 %. Other studies also reported lower sensitivity of RIDTs in detection of influenza type B virus compared to influenza type A virus (Lucas et al. 2011). The present study indicates a good agreement between the two methods: RIDT and RT-PCR (kappa ranged from 0.48 for influenza type B to 0.65 for influenza type A virus) and this observation is consistent with the kappa 0.67 and 95%CI 0.56–0.76 in a study by Gordon et al. (2011).

This particular RIDT (BD Directigen™ EZ Flu A+B) has been evaluated by Blazguez et al. (2010) for detection of the novel influenza A (H1N1)2009 virus in children. The test showed good sensitivity (70.4 %), specificity (100 %), NPV (76.6 %), and PPV (100 %). Chan et al. (2002) found that the test detects a range of human and animal virus A subtypes, including H5N1 and H9N2 subtypes with high sensitivity (96 %), specificity (99.6 %), PPV (96 %), and NPV (99.6 %) for influenza A virus; the respective values for influenza B viruses were: 87.5, 96.8, 80, and 98 %.

The accuracy of different RIDTs was examined in other studies providing different, sometimes opposite results. Ganzenmueller et al. (2010) found that the Quidel QuickVue is not suitable for the diagnosis of infections caused by influenza A(H1N1) 2009 virus and DIA is a superior method. Stevenson and Loeffelholz (2010) also pointed to a poor accuracy of the Quidel Quick Vue test and Cheng et al. (2010) found the same in regard to the Rat Espline test. Hawkes et al. (2010) found sensitivity of RIDT BinaxNow influenza A and B kit (Inverness Medical, Montreal, Quebec, Canada) for detection of A(H1N1) 2009 infections higher in children than that reported among mixed adult-pediatric populations, but still remaining suboptimal. On the other side, a good diagnostic value of RIDT Quidel Quick Vue test has been reported by Lee et al. (2011) (sensitivity 70 %, specificity 97.5 %, PPV 97.4 %, NPV 71.2 %) and Louie et al. (2010) (sensitivity 66 %, specificity 84 %, PPV 84 %, NPV 64 %). Discrepancies in the results regarding RIDTs' accuracy above outlined may stem from the differences in sample types, age of patients studied, duration of symptoms, and viral spreading.

Despite moderate sensitivity for A(H1N1) pdm09 virus detection, the present results suggest that BD Directigen EZ Flu A+B® might be useful as a screening tool for the diagnosis of influenza

for children who are consulted in an outpatient setting due to symptoms of acute respiratory tract infection (Angoulvant et al. 2011). Potentially, the most important aspect of this rapid test is that it can provide timely, accurate and useful information for clinicians. The information can be provided in a real time when the diagnostics, isolation, and therapeutic questions need to be addressed and solved on the spot. However, clinicians must be aware of the limitations of RIDT and test results should be interpreted cautiously. During epidemic or pandemic seasons, false negative results occur more often than false positive results. The physician should consider forwarding respiratory specimens of patients with negative results for influenza obtained in RIDT testing to further RT-PCR, viral culture, or DIA verification, especially when community influenza activity is high and when the patient is at risk for a severe course of disease. For children at high risk for influenza-like illness during high-prevalence periods of influenza, empiric initiation of antiviral therapy should be considered even in case of negative RIDT results (Faix 2009).

One advantage of our study is a homogenous group of patients (children younger than 59 months), while most other studies dedicated to the evaluation of rapid influenza tests have been conducted in adult, mixed adult and pediatric patients, or among patients aged 0–18 years (Noyola and Demmler 2000). We chose young children for the study because the incidence of influenza, the risk of complications, and influenza-related hospitalizations are all high in this age-group (Principi et al. 2004; Neuzil et al. 2002). Another advantage seems to be that the study was conducted in an outpatient setting, while most other studies have been performed in hospitals or emergency units (Nitsch-Osuch et al. 2013; Poehling et al. 2011). A disadvantage, however, could be a relatively small number of patients enrolled, and a small number of positive results for influenza type B infection. Our findings are consistent with those reported in literature in that they confirm that the immunoassay is a specific, but not very sensitive method, in the diagnosis of influenza. Nevertheless, the rapid BD Directigen™ EZ Flu A+B test may be recommended for initial screening for influenza in primary care settings.

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

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Virological Monitoring of Influenza Activity and Influenza-Like Illness in the Epidemic Season 2011–2012 in Poland

12

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Abstract

Influenza and influenza-like illnesses are recorded in all latitudes and in every age group. In Poland, the number of cases varies between several thousand and several million depending on the epidemic season. These figures are probably underestimated since a great deal of patients avoids consulting the doctor. To some extent, this situation is caused by the fear of financial loss resulting from being on sick leave. Influenza virus is classified into three types A, B, and C according to antigenic differences in their nuclear and matrix proteins. Influenza viruses are characterized by their high changeability in terms of hemagglutinin (HA) and neuraminidase (NA). The changes may be referred to as antigenic drift that consists of point mutations in the genes encoding the HA and NA or sudden changes, referred to as antigenic shift that results from an exchange of gene segments encoding hemagglutinin and neuraminidase. Since there is an animal reservoir of influenza type A virus, reassortment of different subtypes of this virus may occur with type A virus strains which occur solely in the human. This can result in the creation of an entirely new strain with hemagglutinin and/or neuraminidase subtypes which have not been encountered in humans previously, to which a large part of the population will not be resistant and which therefore has a pandemic

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_12,

potential. Poland participates in the Global Influenza Surveillance System for influenza and influenza-like infection throughout the year and also during the epidemic season. The main objective of supervision is a continuous monitoring of the influenza situation in the country and the most rapid detection of the emergence of a new strain of influenza virus with pandemic potential.

Keywords
Antigenic drift • Antigenic shift • Influenza-like virus • Influenza virus • Epidemiological surveillance • Mutations

1 Introduction

Since 2001, Poland has been a member of the European Influenza Surveillance Network of the European Center for Disease Prevention and Control (website EISN 2012; Machala et al. 2006b). The country is represented by the Department of Influenza Research, National Influenza Center (DIR-NIC) which is an integral part of the National Institute of Public Health – National Institute of Hygiene (NIPH-NIH), based in Warsaw. The Sentinel influenza surveillance system has operated in Poland since the epidemic season 2004–2005 and is an organization operating independently of the WHO Global Influenza Surveillance and Response System (GISRS) in which Poland has participated for many years. It is believed that the Sentinel system is of key importance in view of the increasingly realistic probability of another influenza pandemic. This paper presents data on cases of influenza and influenza-like illness in the 2011–2012 season.

The paper describes the operation of the Sentinel system using the example of the 2011–2012 epidemic season. This system is used to monitor the incidence of influenza and influenza-like illness in Poland. It also includes the contribution of the Department of Influenza Research, National Influenza Center in this regard. This facility constantly monitors and surveys data on the current activity of the influenza virus at the country level, forwarded from the Voivodeship Sanitary-Epidemiological Station (VSESs), WHO and EISN websites. In addition the present paper, demonstrates the virological situation concerning influenza and influenza-like infection in Poland for the 2011/2012 epidemic season with analysis of data for the period 29.08.2011–24.06.2012 (Table 12.1).

2 Sentinel Influenza Surveillance System in Poland

The Sentinel influenza surveillance system became operational in Poland from the 2004–2005 epidemic season. Its main objective was to integrate epidemiological and virological surveillance in such a way that information on the number of cases of influenza would be associated with laboratory confirmation of infection caused by influenza virus and influenza-like virus. This action was necessary for Poland to join, to the greatest possible extent, the system that already operated in other European countries. That also has made it possible to provide the information required which in terms of its quality and quantity became an integral part of European data on the influenza activity in Poland in comparison with other European countries. Efforts to standardize the influenza surveillance system were supported from 2003 by the National Influenza Center, Influenza Laboratory based at the National Institute of Health, now the National Institute of Public Health-National Institute of Health. It was there that the procedures were developed due to which the Sentinal influenza surveillance system operates today with good results. Specific forms are used for the collection of epidemiological and virological data and for the data on patients with suspected influenza virus infection, from whom samples were taken for virological tests.

Table 12.1 Virological data on the total number of samples collected in the 2011–2012 season per voivodeship region and the number of positive samples

No.	Voivodeship region	All samples collected in the 2011/2012 season[a]	Positive samples obtained in the 2011/2012 season[a]
1	North-East	65	9
2	North-West	1,071	183
3	Central	246	57
4	South-East	529	146
5	South-West	84	7
	Total	1,995	402

[a]Data for the period 29.08.2011–24.06.2012 (week 35 of 2011 to week 25 of 2012)

The National Influenza Centre, Influenza Laboratory, currently the DIR-NIC conducts hands-on laboratory training for laboratory scientists from the Voivodeship Sanitary – Epidemiological Station (VSESs), whose strong commitment is necessary for the proper functioning of the Sentinel system. These courses are primarily a compendium of knowledge on the methods of laboratory detection of influenza virus genetic material and of methods of culture and isolation of influenza viruses from clinical samples.

Surveillance of influenza is conducted throughout the year. It requires special support during the epidemic season, i.e., from about early September to early June. Participants include selected family physicians, the above mentioned VSES which receive the transmitted data from the District Sanitary – Epidemiological Stations, the DIR-NIC which has the role of coordinator and the Department of Epidemiology, based at the NIPH-NIH (Machala and Brydak 2006; Brydak et al. 2005; Machala et al. 2006b).

3 Principles of Operation of Integrated Sentinel Epidemiological and Virological System

A comprehensive system of epidemiological and virological surveillance is focused on a constant, unchanging population which is representative of the whole of the country. Approximately 1–5 % of all physicians in the country and all 16 VSES are involved in this type of analysis. Surveillance figures include recorded new cases of influenza and influenza-like illness for the 0–4, 5–14, 15–64, and $\geq$ 65 years age groups for each of the 52 weeks of the year (according to the numbering of calendar weeks) starting from Monday to Sunday each week, reported by the physicians participating in the program. The data are sent to the appropriate territorial VSES with data on the number of patients assigned to a physician participating in the program (Romanowska and Brydak 2007; Brydak and Machala 2006). Based on the data obtained from family physicians, each of the 16 VSES prepare a summary report for the given week of the year and forward this within the deadline to the DIR-NIC, where the weekly report is prepared for the whole country. This information is then transmitted to the European Influenza Surveillance Network (EISN 2012).

Virological surveillance involves, as also does epidemiological surveillance, the same physicians who are also required to collect samples from patients with influenza and influenza-like symptoms. Samples are sent to the appropriate territorial VSES and to the DIR-NIC. A scheme of the Sentinel integrated viral epidemiological surveillance in Poland is shown in Fig. 12.1.

This monitoring includes:

- Isolation of influenza virus strains and diagnostics using molecular biology techniques and serological analysis of samples

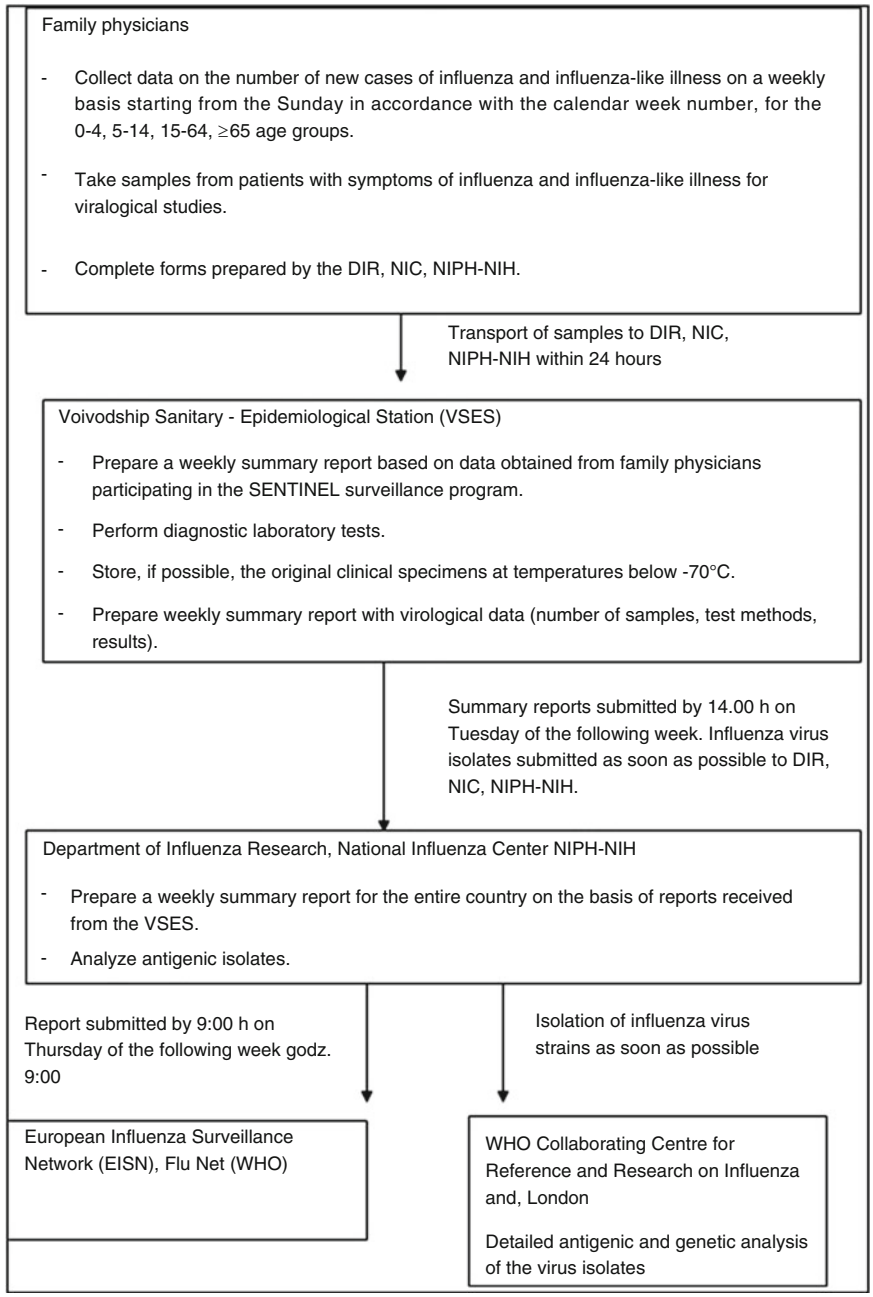

Fig. 12.1 Sentinal viral epidemiological surveillance in Poland

taken from patients with symptoms of influenza or other influenza-like infections.

- Transmission of information concerning the results of virological and serological investigations within the deadline set by the VSES to the DIR-NIC in weekly reports.
- Transfer to the DIR-NIC of influenza virus strains isolated by the VSES or from other institutions. Further analysis of the antigenic and/or genetic strains acquired is conducted by the Department in order to identify the type and subtype.

- The isolated influenza virus strains, including those for which the type/subtype cannot be determined are sent by the DIR-NIC to the WHO Collaborating Center for Reference and Research on Influenza, National Institute for Medical Research (NIMR) in London.
- A report for the whole country is prepared by the DIR-NIC based on own data and those obtained from the reports received each week from the VSES. The report is transmitted to the EISN.

4 Participation in Sentinel Program: Benefits for Doctor and Patient

As in most other countries, participation by family medicine specialists in the Sentinel program is voluntary and is conducted on an honorary basis. Participation in the program has specific benefits for both doctors and patients. For the patient, these include free of charge laboratory testing for influenza and often for other respiratory infections caused by viruses which cause influenza-like symptoms (reimbursement by the National Health Fund). In addition, thanks to the fast availability of results it is possible to administer appropriate and effective treatment to the patient with antiviral and anti-influenza medication. This helps significantly to reduce the duration of the illness with implications for absenteeism from work due to reduced costs for patients and employers, which otherwise would be incurred due to inefficiency of a potentially non-specific treatment of patients (Madej-Pilarczyk et al. 2006; Machala et al. 2006a; Brydak and Machala 2006).

5 Participation in Sentinel Program: Benefits for Country and World

The integrated epidemiological and virological surveillance system for influenza provides data on the activity of influenza and other respiratory viruses not only at the local level, but also for the whole country. Collecting this type of data is important for early warning in cases of epidemic and pandemic influenza in Poland, neighboring countries, and for the world. Incorporating the influenza surveillance system with other countries makes it possible to analyze the epidemiological and virological situation of influenza in Poland in comparison with Europe and the world. These data are supplied to the EISN by the countries participating in the project and provide the basis for the Euro surveillance weekly newsletter, available in an electronic format. The newsletter contains data on the epidemiological and virological situation of influenza in Europe with comments by representatives of selected countries and by the newsletter's editorial team and is available on the website http://ecdc.europa.eu.

6 Incidence of Influenza and Influenza-Like Illness in Poland in 2011–2012

The annual cost of a large outbreak of influenza in Poland can be as high as five billion PLN, and in the capital city of Warsaw alone this cost may reach 600 million PLN, at the minimum, every season due to influenza. It is estimated that 286 million PLN loss results from the illness-related absenteeism, of which 2/3 is caused by influenza itself, and 1/3 results from its complications. Approximately 145 million PLN is expended annually on care of young children. However, 187 million PLN is loss associated with death or long-term absence from work as a result of complications of influenza. Data in Tables 12.1

Table 12.2 Number of samples taken for testing by physicians participating in the SENTINEL influenza surveillance in Poland and the number of samples coming from outside the Sentinel system

Number of samples taken	Epidemic season	
	2004–2005	2011–2012
Sentinel system	399 (91 %)	477 (24 %)
External to sentinel	39 (9 %)	1,518 (76 %)
Total	438	1,995

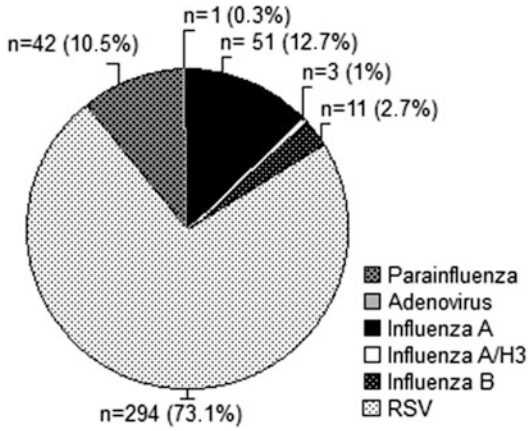

Fig. 12.2 Laboratory confirmation of infections caused by influenza and influenza-like virus causing respiratory disease in Poland for the 2011/2012 epidemic season from Week 35 of 2011 to Week 25 of 2012 (29.08.2011–24.06.2012)

and 12.2 and in Fig. 12.2 indicate that taking into account solely the ever improving operation of the Sentinel influenza surveillance system it is possible to conclude that in the 2011–2012 season nearly 2,000 samples were collected of which over 20 % tested positive for detection of influenza virus or other respiratory virus. Furthermore, the work carried out over the years to improve the Sentinel system can be observed in an increased number of samples taken from patients in the 2011–2012 season compared with the same period in 2004–2005. Data on this issue are presented in Table 12.2.

7 Conclusions

Conducting effective influenza surveillance is not an easy task, and certainly will not be easy during an epidemic or pandemic. However, when comparing the data from the initial period when Poland entered the Sentinel program in the 2004–2005 season with data from 2011 to 2012, it is evident that the awareness of physicians, patients, and the sanitary and epidemiological monitoring services have all considerably increased. The necessity for wide-ranging efforts in terms of monitoring of influenza and influenza-like virus infections has become a reality, without which the modern virologist and epidemiologist would be deprived of tools for the analysis of the current epidemiological and virological situation in Poland in comparison with the situation in Europe and the rest of the world.

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

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Incidence of *Chlamydophila Pneumoniae* Infection in Children During 2007–2010

13

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Abstract

Chlamydophila pneumoniae is the etiological agent of pharyngitis, bronchitis, and pneumonia. The aim of the study was to evaluate the frequency of chlamydial respiratory infections in children in the Lower Silesia Region in Poland in 2007–2010. There were 2,733 throat swabs examined, obtained from hospitalized patients aged from 20 months to 18 years with various clinical symptoms such as dry cough, productive cough, and from asymptomatic ambulatory patients. An indirect immunofluorescence technique, based on monoclonal antibodies labeled with fluorescein isothiocyanate, was used for detection of *Chl. pneumoniae* antigen. Overall, there were 1,114, 503, 641, and 475 patients studied in the consecutive 2007, 2008, 2009, and 2010 years. There clearly were fewer patients each next year submitted for *Chl. pneumoniae* detection procedure, which stemmed from the declining severity of respiratory infections noted in children and thus less demanding diagnostic workup commissioned by physicians. The percentage of results positive for *Chl. pneumoniae* antigen amounted to 53.3, 41.6, 43.1, and 36.4 % in the consecutive years, respectively. Detection of chlamydial infections had thus a decreasing tendency in the period studied. There also were decreases in *Chl. pneumoniae* detection rate in cases stratified due to the presenting symptom: dry cough, productive cough, or in asymptomatic cases. A milder course of respiratory infections resulting in a decreased number of children examined for *Chl. pneumoniae* antigen in consecutive years, makes it difficult to draw definite conclusions on the factual incidence rate. Nevertheless, we believe we have shown, from the clinical standpoint, a dropping rate of *Chl. pneumoniae* detection in children with respiratory infections.

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_13,

Keywords
Chlamydophila pneumoniae • Cough • Incidence • Infection • Respiratory tract

1 Introduction

Chlamydophila pneumoniae (*Chl. pneumoniae*) is the etiological agent of pharyngitis, bronchitis, and pneumonia (Roca 2007). Infections are usually accompanied by hoarseness and long lasting dry cough (Choroszy-Krol et al. 2010; Blasi 2004). Cough, often becoming chronic, and bronchial hypersensitivity persist the longest in children under 4 years of age. *Chl. pneumoniae* spreads by droplets and its reservoir are the sick and asymptomatic vectors and the microorganism often participates in asthma pathogenesis (Korppi 2010).

The aim of the present study was to assess retrospectively the incidence rate of chlamydial respiratory infections in children from the Lower Silesia Region in southern Poland during a 4 year period covering 2007–2010. The incidence rate was evaluated as the percentage of positive detections of *Chl. pneumoniae* antigen in throat swabs in the groups of children stratified according the presenting symptom of respiratory infection, such as dry cough or cough with phlegm, and in asymptomatic cases.

2 Methods

The study was performed in accordance with the Declaration of Helsinki for Human Research of the World Medical Association and was approved of by an institutional Ethics Committee. Medical records of 2,733 children, aged from 20 months to 18 years, concerning the results of throat swabs examinations performed in the years 2007–2010 at the Medical University in Wroclaw, Poland were analyzed. All the children lived in the Lower Silesia Region of southern Poland. For the purpose of this evaluation, the patients were stratified according to gender and to the presenting respiratory symptom, dry cough or cough with phlegm, or the lack of presenting symptoms. The stratification scheme and the number of cases in a given group in consecutive years are shown in Fig. 13.1. The children were referred for the investigation by their family doctors. The method consisted of an indirect immunofluorescence technique, based on monoclonal antibodies labeled with fluorescein isothiocyanate (IFA), for detection of *Chl. pneumoniae* antigen (Chlamydia Cel PN-IFT kit, Cellabs Pty Ltd, Sydney, Australia).

3 Results and Discussion

In 2007, the detection rate of *Chl. pneumonia* antigen was 53.3 % (594/1,114) of the samples studied (Fig. 13.2). The positive results were almost evenly distributed between girls and boys. There were 558 girls of whom 308 tested positive and 556 boys of who 286 tested positive, which comes to 55.2 % and 51.4 %, respectively. Dry cough as a symptom was manifest in 522 out of the 1,114 patients and in 303 of the cough cases *Chl. pneumonia* was detected, i.e., 58.0 %. In patients who had cough with phlegm, the percentage of positive results for *Chl.*

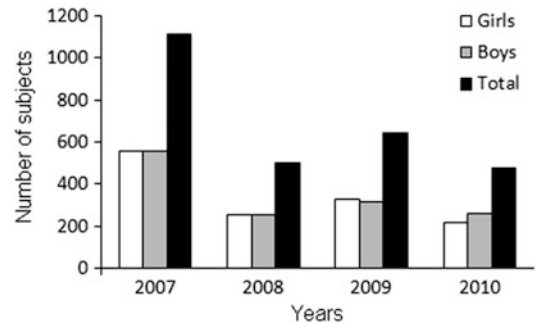

Fig. 13.1 Children examined for the presence of *Chlamydophila pneumoniae* antigen in the consecutive years 2007–2010 – breakdown by gender

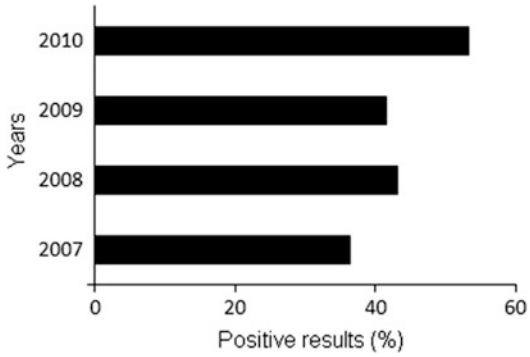

Fig. 13.2 Percentage of positive detection of *Chlamydophila pneumoniae* antigen in all sample examined in the consecutive years 2007–2010

pneumonia was 47.5 % (194/408) of patients. Finally, in the group of children with no symptoms, who were tested prophylactically or just because they had contact with an infected there were 97 children who showed the presence of chlamydial antigen, which accounts for 52.7 % (97/184).

In 2008, the detection rate of *Chl. pneumonia* antigen was 41.6 % (209/503) of the samples studied (Fig. 13.2). Again, there were no appreciable gender differences in infection; 113 of out the 252 girls and 96 out of the 251 boys tested positive, which comes to 44.8 and 38.2 %, respectively. Dry cough was manifest in 218 out of the 503 children, and *Chl. pneumoniae* was detected in 85 (39.0 %) children with this cough. Productive cough with phlegm was present in 188 children of whom 87 (46.3 %) tested positive for *Chl. pneumoniae*. Finally, in the 97 children without symptoms of chlamydial infection, there were 37 (38.1 %) cases of chlamydial antigen detection.

In 2009, samples from 641 children were investigated for the presence of *Chl. pneumoniae* antigen. The results concerning both gender and symptom stratification in the detection rate of the antigen were closely akin to those obtained for the year 2008 above outlined (Fig. 13.2).

In 2010, continuation of the descending trend was noted in both the number of children being commissioned by doctors to the throat sampling procedure and the detection rate of *Chl. pneumoniae* antigen in those subjected to sampling. A total of 475 children were sampled. On the whole, the detection rate of *Chl. pneumoniae* was 36.4 %; there were 173 positive results out of the 475 tests (Fig. 13.2). In the study group, there were 216 girls, of whom 90 (41.7 %) tested positive and 259 boys, of whom 83 (32.0 %) tested positive. The presence of *Chl. pneumoniae* was detected in 89 (37.6 %) cases out of the 237 patients with dry cough, and in 66 (34.6 %) cases out of the 191 patients who presented with productive cough. The group without symptoms of chlamydial infection numbered 47 children, of whom 18 (38.3 %) tested positive for the presence *Chl. pneumoniae*.

In the present study, the overall incidence of chlamydial infection had a decreasing tendency over the consecutive years studied. There also were decreases in *Chl. pneumoniae* detection rate in cases broken down by the presenting symptom: dry cough, productive cough with phlegm, or in asymptomatic cases. Dry stifling cough and hoarseness were the most common symptom of infection (Fig. 13.3). Among the patients with symptoms of dry cough, a downward trend in chlamydial infections was confirmed in 2010. Dry cough often became protracted as a result of chlamydial infection, which was due to hypersensitivity bronchitis, pharyngitis, or sinusitis developing as productive sequelae of the infection. The infection could also take on the form of a carrier state or subclinical asymptomatic state. We also found that girls are more likely to acquire chlamydial infection than boys; this gender difference was seemingly increasing in consecutive years from about 4 % points in 2007 to 10 % points in 2010, although it failed to reach statistical significance (Fig. 13.4). We can offer no readily explanation for a greater sensitivity of female gender for chlamydial infection. A decreased incidence of chlamydial infection should, however, be treated with caution, as it might be, in part, a spurious finding. A milder course of respiratory infections, noted in general, resulted in a decreased number of children examined for *Chl. pneumoniae* antigen in consecutive years. This milder course of infections could reflect an increasingly extensive, general use of macrolides that also could counteract the development of chlamydial infection in children. Thus it

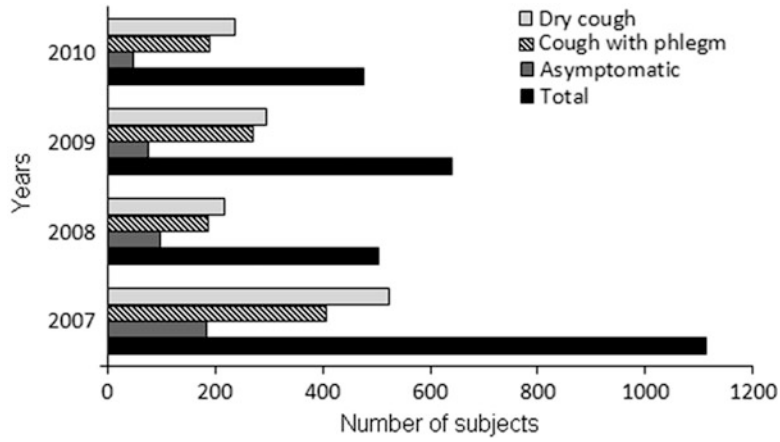

Fig. 13.3 Number of children with positive detection of *Chlamydophila pneumoniae* antigen in the consecutive years 2007–2010 – breakdown by the presenting symptom

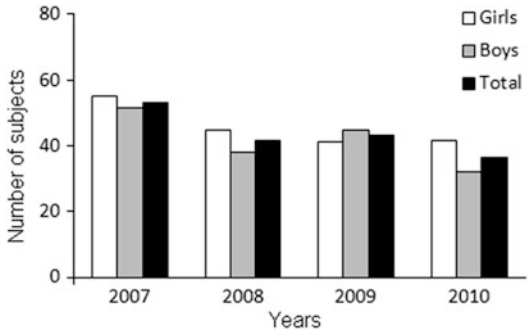

Fig. 13.4 Number of children with positive detection of *Chlamydophila pneumoniae* antigen in the consecutive years 2007–2010 – breakdown by gender

is difficult to draw definite conclusions on the factual incidence rate. Nevertheless, we believe we have shown, from the clinical standpoint, a dropping rate of *Chl. pneumoniae* detection in children with respiratory infections.

According to various studies, upper respiratory tract infections with chlamydial etiology occur in 3–58 % of children aged 3–15 years, 3–33 % of community-acquired pneumonia, and 15–75 % of bronchitis (Zielnik-Jurkiewicz 2008; Korzon 2009). Kumar et al. (2011), examining children with lower respiratory tract infections, have reported chlamydial infection, based on antibodies, in 6 % of patients. Esposito et al. (2004) have shown the presence of *Chl. pneumoniae* in 13 % of children with acute pharyngitis, but the pathogen was a single underlying cause of infection only in 3 % of the cases. Kowalewska-Pietrzak et al. (2011) have studied children aged 4 month to 7 years hospitalized for chronic cough and/or chronic pneumonia. The authors have reported positive results for *Chl. pneumoniae* in about 29 % of the cases. The differences concerning the prevalence of *Chl. pneumoniae* infection above outlined have likely to do with different methodologies employed for the pathogen detection, different diagnostic procedures, and different types of infections. Nevertheless, the literature review undoubtedly points to the presence of a heavy burden of *Chl. pneumoniae* infection, particularly in children; the finding confirmed in the present study, albeit with a decreasing tendency in recent years.

4 Conclusions

The present study demonstrates that the detection rate of chlamydia infections in children showed a downward trend from 53.3 to 36.4 % between 2007 and 2010. *Chlamydophila pneumoniae* is an atypical pathogen, and the diagnosis of this pathogen should be considered after exclusion of other, typical for the patent's age microorganisms affecting the respiratory system. The possibility of chlamydial infection should particularly draw attention in cases presenting with dry cough.

Acknowledgments Supported by Wroclaw Medical University grant ST-718.

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

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Clinical Features and Outcomes of Influenza *A* and *B* Infections in Children

14

Aneta Nitsch-Osuch, Agnieszka Wozniak-Kosek, Krzysztof Korzeniewski, Katarzyna Zycinska, Kazimierz Wardyn, and Lidia B. Brydak

Abstract

The aim of the study was to describe the course of influenza among children aged 0–59 months. A total of 150 children with influenza-like symptoms (ILI): cough, fever >37.8 °C, and sore throat was included into the observation. All children were tested with both rapid influenza detection test (RIDT) BD Directigen™ EZ Flu A+B® and RT-PCR. Sixty four cases of influenza were diagnosed (incidence rate 40 %): 19 (30 %) cases of influenza caused by type B virus and 45 (70 %) cases caused by type A virus. Children with influenza required more often follow up visits ($p < 0.05$, OR 1.99, 95 % CI 1.03–3.85) and less often were administrated antibiotic therapy ($p < 0.05$, OR 0.25, 95 % CI 0.04–0.97). The logistic regression analysis revealed that only positive result of rapid influenza detection test, not any of clinical symptoms, could be found as an independent predictor of influenza (OR 4.37, 95 % CI 2.03–9.43). Patients with influenza type A more often reported muscle ache ($p < 0.05$) and complications ($p < 0.05$; OR 6.06, 95 % CI 1.20–60.38). Otitis media occurred more often among patients with than without influenza ($p < 0.01$; OR 15.50, 95 % CI 2.10–688.5). We conclude that although influenza infections among children younger than 59 months were generally mild and self-limited, pediatric burden of the disease was significant.

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_14,

Keywords
Children • Incidence rate • Influenza • Outcome • Rapid influenza detection test • Virus

1 Introduction

Influenza viruses are an important cause of disease of varying severity in humans. WHO estimates that annual influenza epidemics result in about three to five million cases of illness, and about 500,000–1,000,000 deaths in the global population at large. Typically, the incidence rates during the annual outbreaks of influenza are highest in children, with an average of 20–30 % of the pediatric population affected (Heikkinen 2006). Surveillance studies from several industrialized countries reported an incidence of pediatric hospitalizations associated with seasonal influenza of 11–54/100,000 children <5 years of age, and the incidence of 1–13/100,000 children treated for influenza in pediatric intensive care units (D'Onise and Raupach 2008; Rojo et al. 2006). For the influenza season 2003–2004, 152 influenza-associated deaths among children were reported in the USA; 47 % of them occurred in children without underlying chronic diseases (Bhat et al. 2005).

Clinical manifestations of influenza infections range from illness with asymptomatic, atypical (i.e., gastro-intestinal) or oligosymptomatic diseases to severe toxic progression resulting in death. Complications of influenza may occur at any age, affecting mostly infants and children, patients with chronic underlying diseases as chronic pulmonary diseases, diseases of the circulatory system, metabolic diseases, or immunodeficiency. The most severe complications are acute circulatory failure due to myocarditis or fulminant necrotizing influenza-associated pneumonia. A frequently reported complication is a secondary bacterial pneumonia, other known complications include encephalitis, otitis media, and neuromuscular diseases (Dilantika et al. 2010; Rothberg et al. 2008; Quach et al. 2003).

The aim of the present study was to describe the course of influenza caused by viruses type A and B among children aged 0–59 months consulted in the ambulatory settings.

2 Methods

The study was approved by a Local Ethics Committee (No KB 165/2011). A total number of 150 children aged 0–59 months with influenza-like symptoms (ILI): cough, fever >37.8 °C, sore throat was included into the observation in the two consecutive influenza seasons: 2009/2010 and 2010/2011. The inclusion criteria for the study were: age less than 59 months, presentation of influenza-like illness according to CDC definition (fever >37.8 °C, cough and/or sore throat in the absence of another known cause of illness), duration of symptoms shorter than 96 h (4 days). The exclusion criteria were: age older than 59 months, duration of symptoms longer than 96 h, and current antibiotic or antiviral therapy.

All children were tested with both rapid influenza detection test (RIDT) BD Directigen™ EZ Flu A+B (Becton, Dickinson and Company, Sparks, MD) and RT-PCR. The samples positive in RT-PCR were additionally tested by real time RT-PCR to diagnose the subtype of influenza A virus. The RIDT was conducted by previously trained medical staff and the results were obtained within 15 min. Specimens were processed on-site at the general practitioner's office according to the manufacturer's recommendations. The biological material for testing was obtained from two nasal and one pharyngeal swabs. Positive and negative test results of RIDT were determined with the use of a visual key provided with the test kits. The RT-PCR and real time RT-PCR were conducted by the specialized staff in the National Influenza

Center in Warsaw, Poland. Isolation of viral RNA was conducted with Maxwell® 16 Viral Total Nucleic Acid Purification Kit (Promesa Corp., USA). One-step RT-PCR was conducted with Transcriptor One-step RT-PCR Kit (Roche Diagnostics, Switzerland). Real time RT – PCR was conducted with Invitrogen SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (Invitrogen, USA) using CDC Real-time RTPCR (rRTPCR) (WHO Protocol for Detection and Characterization of Swine Influenza 2009).

Comparisons between the following groups were performed: (a) the groups with laboratory confirmed and excluded influenza, (b) the groups with influenza caused by virus type A and B. We used a nonparametric Mann–Whitney *U* test for comparisons of age and duration of symptoms. The Chi^2 or Fisher's Exact Test for Count Data was used to compare categorical variables between groups. Logistic regression analysis was conducted to identify independent risk factors for diagnosis of influenza. The results are presented as odds ratios (OR) with 95 % confidence intervals (95 % CI). $P < 0.05$ was considered statistically significant. All statistical analyses were performed using a commercial SPP packet (ver. 15.0 for Windows, Chicago, IL).

3 Results

According to RT-PCR results, a total number of 64 cases of influenza was diagnosed (incidence rate 40 %): 19 (30 %) cases of influenza caused by virus type B and 45 (70 %) cases caused by virus type A. Real time RT-PCR revealed that all cases of influenza A were caused by subtype A (H1N1)pdm09.

There were no significant differences between the laboratory confirmed and excluded influenza concerning the presenting clinical symptoms, duration of symptoms prior to the consultation with GP, treatment with over-the-counter medicines administrated by parents to their children, influenza vaccination status, and underlying diseases (Table 14.1). However, children with laboratory confirmed influenza required significantly more often follow-up visits ($p < 0.05$, OR 1.99, 95 % CI 1.03–3.85) and less often were administrated antibiotic therapy ($p < 0.05$, OR 0.25, 95 % CI 0.044–0.97). Only two children with confirmed influenza received antiviral treatment with oseltamivir, both of them had an underlying medical condition (bronchial asthma) known to be a risk factor for influenza. Treatment with oseltamivir was administrated for 5 days, no complications of influenza, and no side effects of oseltamivir were observed. Otitis media as a complication of influenza-like symptoms was more often reported among children with influenza compared to children without influenza ($p < 0.01$, OR 15.5, 95 % CI 2.1–688.56) and was more often diagnosed among children with influenza A than influenza B ($p < 0.05$).

The accuracy of individual symptoms of influenza was insufficient to help in the diagnosis (Table 14.2). The results of logistic regression analysis revealed that only a positive result of rapid influenza detection test, not any of the clinical symptoms, was an independent predictor of influenza (OR 4.37, 95 % CI 2.03–9.43) (Table 14.3). However, true positive results of RIDT were obtained only in 55 % cases of influenza confirmed by RT-PCR.

Comparing the outcome and clinical features of influenza caused by virus A(H1N1)pdm09 and virus B we did not reveal any appreciable differences between the age of patients, frequency of underlying conditions as the risk factors for severe influenza, vaccination status, symptomatic treatment before consultation with GP. However, we observed that patients with influenza A(H1N1)pdm09 significantly more often reported muscle ache than patients with influenza B ($p < 0.05$). Another observation was that the parents of children with laboratory confirmed influenza B were consulted by GP 72 h after the onset of symptoms more often than those of children with influenza A(H1N1) pdm09 ($p < 0.05$, OR 0. 27; 95 % 0.06–1.13). Complications of influenza were more likely to appear in the children with influenza A(H1N1)

Table 14.1 Medical characteristics and underlying conditions of patients with laboratory confirmed and excluded influenza

	Number of patients with influenza	Number of patients without influenza	p	OR (95 % CI)
Underlying condition as a risk for severe influenza	10[a]	9[b]	0.35	1.58 (0.60–4.10)
Vaccination against influenza in current season	1	2	–	0.66 (0.01–13.10)
Age of patients (months)				
<12 m	1	6	0.23	0.21 (0.004–1.82)
12–24 m	12	22	0.32	0.67 (0.3–1.48)
>24 m	51	58	0.01	1.89 (0.88–4.04)
Duration of symptoms before consultation with GP (hours)				
<24 h	6	8	0.99	1.0 (0.33–3.06)
24–48 h	26	36	0.88	0.95 (0.49–1.83)
48–72 h	19	26	0.94	0.97 (0.48–1.97)
72–96 h	13	16	0.49	1.11 (0.49–2.52)
Symptoms				
Cough	59	84	0.13	0.28 (0.03–1.80)
Sore throat	60	80	1	1.12 (0.25–5.66)
Sneezing	54	78	0.24	0.55 (0.20–1.49)
Weakness	44	53	0.37	1.36 (0.69–2.71)
Headache	17	23	0.98	0.99 (0.47–2.05)
Muscle ache	11	15	0.97	0.98 (0.41–2.31)
Gastrointestinal	11	8	0.15	2.02 (0.76–5.30)
Symptomatic treatment before consultation				
Antipyretics	57	72	0.35	1.58 (0.59–4.10)
Mucolytic syrups	18	34	0.15	0.59 (0.29–1.19)
Anticough syrups	4	6	1	0.88 (0.17–3.94)
Nasal congestive drops and sprays	15	30	0.13	0.57 (0.27–1.18)
Hospitalization	0	2	0.22	–
Follow-up visit (at least one)	37	35	0.04	1.99 (1.03–3.85)
Additional tests (blood, urine, others)	6	10	0.66	0.78 (0.27–2.28)
Antibiotic therapy	3	14	0.04	0.25 (0.04–0.97)
Antiviral therapy	2	0	–	–
Complications (in total)	21	11	0.01	3.09 (1.35–7.06)
Acute otitis media	10	1	0.001	15.5 (2.1–688.6)
Pneumonia	9	7	0.25	1.84 (0.60–2.50)
Bronchitis	1	3	0.63	0.44 (0.01–5.64)
Laryngitis	1	0	0.42	–

[a]9 children with bronchial asthma, 1 child with hemophilia
[b]8 children with bronchial asthma, 1 child with hemophilia

Table 14.2 Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LH+), negative likelihood ratio (LH−) of symptoms of influenza

Symptom	Sensitivity (95 % CI)	Specificity (95 % CI)	PPV (95 % CI)	NPV (95 % CI)	LH+ (95 % CI)	LH− (95 % CI)
Cough	92.2 %	2.3 %	41.3 %	28.6 %	0.94	3.35
	(90.0–96.0)	(1.0–50.0)	(40.0–43.0)	(8.3–63.4)	(0.96–1.01)	(0.77–15.0)
Sore throat	93.8 %	7 %	42.9 %	60 %	1.01	0.90
	(89.4–97.3)	(3.7–9.6)	(40.9–44.5)	(32–82.9)	(0.93–1.08)	(0.28–2.86)
Sneezing	84.4 %	9.3 %	40.9 %	44.4 %	0.93	1.68
	(79.0–90.2)	(5.0–13.0)	(38.3–43.7)	(25.4–65.2)	(0.83–1.05)	(0.72–3.96)
Weakness	68.8 %	38.4 %	45.4 %	62.3 %	1.12	0.81
	(59.9–77.1)	(31.8–44.6)	(39.5–50.9)	(51.5–72.3)	(0.88–1.39)	(0.52–1.26)
Headache	26.6 %	73.3 %	42.5 %	57.3 %	0.99	1.00
	(18.8–34.8)	(67.5–79.3)	(30.1–55.6)	(52.8–62.0)	(0.58–1.68)	(0.82–1.20)
Muscle ache	17.2 %	82.6 %	42.3 %	57.3 %	0.99	1.00
	(10.8–24.2)	(77.8–87.7)	(26.6–59.4)	(54.0–60.0)	(0.48–1.98)	(0.68–1.15)
Gastrointestinal	17.2 %	90.7 %	57.9 %	59.5 %	1.85	0.91
	(11.1–22.6)	(86.2–94.7)	(37.5–76.1)	(56,5–62.2)	(0.81–4.27)	(0.,82–1.03)

Table 14.3 Clinical symptoms and results of rapid influenza detection test as independent predictors of influenza-logistic regression analysis

Symptom	OR	95 % CI
Sneezing	0.68	0.48–0.96
Cough	1.41	0.78–1.96
Sore throat	0.75	0.54–1.05
Muscle ache	0.73	0.34–1.60
Headache	0.74	0.40–1.38
Gastrointestinal	1.5	0.61–3.67
Weakness	0.84	0.55–1.24
Positive result of RIDT	4.38	2.03–9.43

pdm09 ($p < 0.05$; OR 6.06, 95 % CI 1.2–60.38) (Table 14.4).

4 Discussion

The incidence rate of influenza in our study population limited to children younger than 59 months was 43 %; our results are in agreement with other researchers. Tsolia et al. (2006) estimated the frequency of influenza among children younger than 2 years with ILI at 31.7 %, while among patients aged 2–5 years it was 41.5 %. De La Rocque et al. (2009) observed that 41.7 % of children with ILI consulted in the primary care settings had positive results of RIDT (the mean age of patients was 4.1 years). Influenza was also diagnosed in 56.8 % of children aged 1–12 years with ILI consulted in the ambulatory care settings during the winter season of 2009 (Jennings et al. 2009). The 40 % incidence rate found in the present study could have been underestimated because we used fever, temp. >37.8 °C, as one of the inclusion criteria and it was likely that some cases of influenza presented without or lower fever were missed. However, our results strongly confirm the necessity of taking influenza under consideration as a cause of fever and respiratory tract symptoms among children younger than 59 months.

In the present study, influenza, both caused by type A(H1N1)pdm09 and B virus, was generally self-limited and mild. No children with laboratory confirmed influenza required hospitalization, no deaths were reported. Only did two children (one each of either influenza virus) receive antiviral treatment with oseltamivir; both of them also suffered from bronchial asthma, a known risk condition for a severe course of influenza. Both children recovered without complications and no side effects of oseltamivir were observed. A decision to introduce treatment with oseltamivir was taken late – after 72 h of symptom onset, indicating that the diagnosis of influenza should have been taken

Table 14.4 Medical characteristics and underlying conditions of patients with confirmed influenza type A and type B

	Number of patients with influenza A	Number of patients with influenza B	p	OR (95 % CI)
Underlying condition as a risk for severe influenza	8[a]	2[b]	0.70	1.82 (0.31–19.40)
Vaccination against influenza in current season	0	1	0.20	–
Age of patients (months)				
<12 m	1	0	1	–
12–24 m	11	1	0.09	0.09 (0.72–26.00)
>24 m	33	18	0.08	0.08 (0.003–1.22)
Duration of symptoms before consultation with GP (hours)				
<24 h	5	1	0.66	2.22 (0.22–112.18)
24–48 h	22	4	0.05	3.50 (0.92–16.85)
48–72 h	12	7	0.55	0.62 (0.17–2.34)
72–96 h	6	7	0.04	0.27 (0.06–1.13)
Symptoms				
Cough	43	16	0.15	3.82 (0.41–51.10)
Sore throat	42	18	1	0.78 (0.01–10.49)
Sneezing	38	16	1	1.01 (0.15–5.20)
Weakness	30	14	0.76	0.71 (0.16–2.65)
Headache	14	3	0.35	2.37 (0.54–14.77)
Muscle ache	11	0	0.02	–
Gastrointestinal	9	2	0.48	2.10 (0.37–22.00)
Symptomatic treatment before consultation				
Antipyretics	40	17	1	0.94 (0.08–6.40)
Mucolytic syrups	12	6	0.76	0.79 (0.21–3.13)
Anticough syrups	4	0	0.30	–
Nasal congestive drops and sprays	8	7	0.10	0.37 (0.09–1.90)
Hospitalization	0	0	1	–
Follow-up visit (at least one)	29	8	1	1.10 (0.35–3.62)
Additional tests (blood, urine, others)	2	4	0.04	0.18 (0.01–1.40)
Antibiotic therapy	2	1	1	0.80 (0.04–52.10)
Antiviral therapy	1	1	0.50	0.41 (0.01–33.80)
Complications (in total)	19	2	0.02	6.06 (1.20–60.38)
Acute otitis media	10	0	0.03	–
Pneumonia	8	1	0.55	3.82 (0.45–181.97)
Bronchitis	0	1	0.29	–
Laryngitis	1	0	1	–

[a]7 children with bronchial asthma, 1 child with hemophilia
[b]2 children with bronchial asthma

earlier to provide an effective treatment. The question is of whether more children from our study with confirmed influenza should have been treated with oseltamivir. Neuraminidase inhibitors (including oseltamivir and zanamivir) are the drugs of choice for seasonal and pandemic influenza caused by type A(H1N1) pdm09 virus. However, antiviral treatment is not recommended for all patients with confirmed or suspected influenza. The United States Centers for Disease Control and Prevention (2009) released updated guidelines on the treatment of novel influenza A(H1N1)pdm09, which recommend the treatment for children with clinical or radiological signs of pneumonia, or for those who present with severe dehydration, renal or multiorgan failure, rhabdomyolysis, myocarditis, septic shock, or encephalopathy.

Children with exacerbations of underlying chronic disease requiring hospitalization should also be treated. Antiviral treatment is not recommended for all children with influenza because of the risk of resistance. It has been reported that 5.4 % of influenza cases in children between ages of 1 and 12 years are resistant to oseltamivir (Nitsch-Osuch et al. 2008). Shin et al. (2011) showed that more than half of their patients who were resistant to oseltamivir were younger than 59 months of age. That suggests that young age may be a risk factor for infection with a drug-resistant strain and may explain a protracted course of influenza, longer viral shedding, and higher viral titters.

The present study shows that it is not possible to differentiate between influenza and other respiratory tract diseases with influenza-like symptoms from the presenting clinical signs and symptoms. There was no difference between clinical symptoms of influenza among patients infected with influenza A and B virus; except the muscle ache being more likely reported by patients with influenza type A infection. These findings are in line with those by others (Esposito et al. 2011b; Daley et al. 2000). Only can a positive result of RIDT be consider a predictor of influenza, which indicates that a rapid influenza detection test should be more widely used for the early diagnosis among ambulatory care patients. Nevertheless, RIDT should be interpreted with caution due to possible false negative results.

In the present study patients with influenza B were later seen by GPs and less often complained of muscle ache than those with influenza A ($p < 0.05$) which may be considered as indirect sign of less severe symptoms of the disease. Also, complications of influenza were diagnosed more often among children with influenza A compared to influenza B; the most common complication was otitis media. Thus, we confirm the observations of other authors that the course of influenza B may be milder than that of influenza A in the general pediatric population (Mall et al. 2011; Gutierrez et al. 2011; Shiley et al. 2010; Chi et al. 2008). We failed to note an appreciable difference in the necessity of follow-up visits between patients with influenza A and B infection. Such a difference, however, was noted between patients with confirmed and excluded influenza. A greater number of follow-up visits recommended for children with laboratory confirmed influenza may increase indirect costs of disease for the parents due to loss of working days as previously described by Esposito et al. (2011b). It should be noted that our present study has got a limitation stemming from a relatively small number of cases of influenza, especially type B, which limits the power to detect differences in the characteristics and clinical presentation, particularly between influenza A and B infections.

5 Conclusions

Influenza infections among children younger than 59 months are generally mild and self-limited. Nevertheless, pediatric burden of the disease is significant. Our data add up to current discussions on the need to increase the influenza vaccine coverage among children and adolescents in Poland. Up to now, the influenza vaccine coverage among children less than 5 years has been at a low rate below 2 % (Nitsch-Osuch et al. 2010).

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

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Respiratory Complications in Children Hospitalized with *Varicella*

15

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Abstract

The aim of the study was to retrospectively determine the incidence and clinical course of varicella-related respiratory complications in children during the 6-year period 2005–2010. We attempted to identify the predisposing factors and outcome of such complications. Clinical records of 237 children treated in an academic hospital of the Medical University in Wroclaw, Poland were reviewed, taking into consideration the reason for referral to the hospital, duration of hospitalization, and diagnosis. There were 28 (11.8 %) children (mean age 2.8 ± 2.8 years) in the cohort hospitalized with varicella-related respiratory complications. The infants younger than 1 year predominated (9/28). None of the children were previously immunized against varicella. Admission occurred 5.0 ± 2.8 days after the first symptoms of varicella. The source of infection was an older sibling in 13/28 cases. The mean duration of hospitalization was 5.4 ± 2.0 days. The main symptoms were fever (20/28), cough (26/28), tachypnea (11/28), and dyspnea (7/28). Chest X-ray was performed in eight children, confirming pneumonia in six cases. Based on blood gases, chest X-ray, and clinical symptoms, pneumonia was diagnosed in 15/28 and acute bronchitis in 8/28 children. Intravenous antiviral therapy with acyclovir was administered in 16/28 and antibiotics in 14/28 children. In two cases, oxygen therapy was required and one child presented respiratory failure treated in the Intensive Care Unit. We conclude that respiratory tract involvement in the course of varicella infection in children is relatively common. Age less than 1 and an infected older sibling seem major risk factors for respiratory complications.

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DOI 10.1007/978-94-007-6627-3_15,

Keywords
Bronchitis • Cocoon strategy • Immunization • Pneumonia • Respiratory complications • Varicella • VZV

1 Introduction

Varicella, commonly known as chickenpox, is the most frequently reported infectious disease in Poland. In 2011, the incidence of the disease was 452.7 per 100,000 inhabitants (National Institute of Public Health – PZH 2012). The infection is commonly, but erroneously, perceived as a mild childhood disease and the saying that 'every child should get it, the sooner the better' is popular. Although usually mild, complications, such as bacterial skin and soft tissue infections, and the involvement of neurological and respiratory systems do occur, especially in high-risk groups, and lead to a substantial number of hospitalizations (Atkinson et al. 2012). In recent years, varicella also often occurs in adults (Choo et al. 1995), with a more severe course and complications such as viral or bacterial pneumonia (Alanezi 2007; Avnon et al. 2009). So called 'varicella pneumonia' in healthy adults a mortality rate of 10–30 % (Centers for Disease Control and Prevention – CDC 1997), although recent data show that mortality due to varicella complications significantly decreases, probably as a result of antiviral therapy with acyclovir (Chiner et al. 2010). The risk of life-threatening pulmonary disorders is about 25 times lower in children than in adults (Mohsen and McKendrick 2003; Avnon et al. 2009), and there are only several studies describing respiratory complications of varicella in children, with missing data on the subject from Poland. Pneumonia is considered a major health threat in young children; particularly those less than 5 years of age (Rudan et al. 2008), and antivirals can curb the clinical course of varicella-related pneumonia, which may pose diagnostic difficulties (Edmond et al. 2012). In the present study, therefore, we set out to determine the incidence and clinical course of respiratory complications in children admitted to the hospital due to varicella during the 6 year period 2005–2010. We tried to identify the predisposing factors and outcome in the era of effective antiviral therapy.

2 Methods

We conducted a retrospective review of 237 charts of children aged 0–18, who were hospitalized due to complications of varicella in the Department of Pediatric and Infectious Diseases of Wroclaw Medical University in Wroclaw, Poland in the years 2005–2010. Then, we selected the charts of patients with varicella in whom symptoms were suggestive of respiratory complications of the disease and were reported as the direct causes of hospital admission, i.e., dyspnea and breathing effort, tachypnea, cough, and high fever. We analyzed reasons for referral to hospital, accompanying symptoms, results of laboratory and radiological tests, treatment, and final diagnosis. The duration of hospitalization was also analyzed.

Pneumonia was diagnosed when cough, tachypnea or reduced oxygen saturation, and fever higher than 38.5 °C occurred. Acute bronchitis was defined as cough and abnormal breathing sounds on auscultation, with no previously listed symptoms of pneumonia co-existing. Cough was attributed to varicella if it was absent before the disease and chronic pulmonary diseases, e.g., asthma, were not suspected. Hypoxemia was defined as partial oxygen pressure in the blood lower than 75 mmHg and oxygen saturation reduced below 95 %. In children with pneumonia, with low inflammatory markers (C-reactive protein) and sterile blood culture, viral etiology was presumed. In patients with elevated markers and sterile blood culture, the etiology was regarded as undefined. In calculations, means ± SD and 95 % confidence intervals (CI) were used.

3 Results

There were 28/237 children (11.8 %; CI: 8.0–16.6 %), including 13 females (46.4 %), hospitalized with symptoms of respiratory tract involvement due to varicella infection. The mean age of these patients was 2.8 ± 2.8 years. Infants younger than 1 year predominated (9/28; 32.1 %; CI: 15.9–52.4 %). The distribution of hospitalized children by age is illustrated (Fig. 15.1).

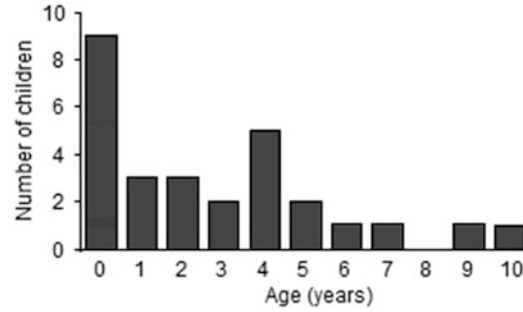

Fig. 15.1 Distribution by age of children hospitalized due to respiratory complications

The source of infection in 13/28 cases (46.4 %), including 8/28 children (28.6 %) younger than 1 year, was an older sibling. There were 5/28 patients (17.9 %) with co-existing chronic diseases, such as atopic dermatitis (3/28; 10.7 %), congenital heart defect (1/28), and juvenile arthritis (1/28). None of the children were previously vaccinated against varicella. We identified the two main risk factors predisposing to a severe course of varicella: age less than 1 in 9/28 (32.1 %) and immunosuppressive therapy in 1/28 patients. Admission occurred 5.0 ± 2.8 days after the first symptoms of varicella. Respiratory tract involvement occurred in 22/28 children (78.6 %) before admission, another 5 patients (17.9 %) developed the symptoms during the hospital stay; in one case the data were missing. The mean duration of hospitalization was 5.4 ± 2.0 days. The main symptoms reported in children suspected of respiratory complications were: fever (20/28; 71.4 %), cough (26/28; 92.9 %), tachypnea (11/28; 39.3 %), and dyspnea (7/28; 25.0 %). Blood gas analysis was performed in 14/28 (50.0 %) patients with dyspnea and hypoxemia was found in all of them. Abnormal breathing sounds on auscultation were detected in 19/28 (67.9 %) patients, including 12 (42.9 %) patients with pneumonia. Chest X-ray was performed in 8/28 (28.6 %) children; pneumonia was confirmed in six of them. Finally, based on blood gases, chest X-rays, and clinical symptoms, pneumonia was diagnosed in 15/237 (6.3 %; CI: 3.6–10.2 %) and acute bronchitis in 8/237 (3.4 %; CI: 1.5–6.5 %) children with complicated varicella. Based on the level of the inflammatory C-reactive protein marker, the etiology of pneumonia was defined as viral in 6/15 cases (40.0 %), with CRP <10 mg/l. In 9/15 (60.0 %) children with pneumonia, the etiology remained undefined. In 5/28 patients (17.9 %) other bacterial complications also occurred and one patient presented neurological symptoms. Cough without bronchitis or pneumonia was found in 5/237 (2.1 %; CI: 0.7–4.9 %) patients (Fig. 15.2).

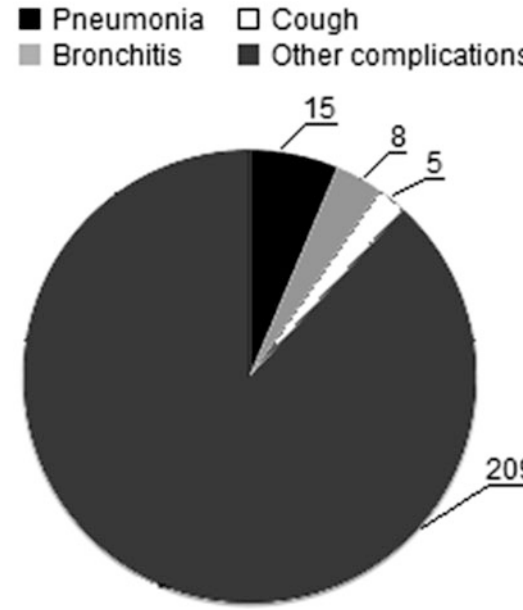

Fig. 15.2 Respiratory complications in children hospitalized with varicella. There were 28 children with respiratory-related symptoms among the total of 237 children hospitalized due to varicella infection

Intravenous antiviral therapy with acyclovir was administered in 16/28 patients (57.1 %), another 5 patients (17.9 %) were given oral

acyclovir. Oral therapy with acyclovir after hospitalization was recommended in 7/28 patients (25.0 %). Antibiotic therapy, most frequently with a third generation cephalosporin, was prescribed in 14/28 patients (50.0 %). In 8/28 cases (28.6 %) both acyclovir and antibiotics were administered. Five patients did not receive either drug. Due to persistent cough and dyspnea, 10/28 (35.7 %) patients received inhalations of β2-agonists and parasympatholytic drugs (fenoterole and ipratropium bromide). In three (10.7 %) cases, inhalative corticosteroids were also administered. In 2/28 patients, oxygen therapy was required. A 5-year-old girl, admitted with pneumonia complicated by septic shock and respiratory failure was transferred to the Intensive Care Unit.

4 Discussion

There are only a few studies describing respiratory diseases as a complication of varicella infection in children. Prospective nationwide studies were carried out in Germany, the UK, and Ireland. The German study included only immunologically healthy children up to 16 years of age, hospitalized due to varicella complications (Ziebold et al. 2001). In that study, various infectious accounted for 38.6 % complications and only one child suffered from pneumonia, which was caused by *Streptococcus pneumoniae*. Other prospective study from the UK and Ireland included 112 children up to 16 years of age, hospitalized with varicella infection (Cameron et al. 2007). Four patients from the group (3 %) were immunocompromised and another 14 had other chronic disease. Pneumonia and bacteremia/septic shock were the most frequent causes of hospitalization (Cameron et al. 2007). Other studies reported rates of pneumonia from 7 to 25 % of children admitted due to varicella complications (Rivest et al. 2001; Piqueras Arenas et al. 2005). Hervás et al. (2011) reviewed the clinical documentation on children hospitalized due to varicella-associated pneumonia in the three hospitals on the Island of Mallorca. Pneumonia was the cause of 8 % of hospital admissions due to varicella infection. The authors also analyzed the etiology of pneumonia based on chest X-rays, laboratory and microbiological tests, and clinical presentation, and found that about half of children (53 %) had pneumonia with bacterial etiology. In our present study, respiratory complications accounted for 11.8 % of hospitalizations due to complicated varicella infection in a 6-year period, but the number of pneumonia cases was 6.3 % (15/237), which is somewhat lower than that in the other studies outlined above.

In the present study, the etiology of pneumonia was viral in 40 % of the patients, which differs from the observations of Hervás et al. (2011), where 9/17 cases of pneumonia were reported as bacterial. In that study, the exact etiology of pneumonia was defined only in 2/9 cases (*Streptococcus pyogenes* isolated from the blood) and in 3 of the remaining 7 cases viral etiology was confirmed – respiratory syncytial virus in two cases and adenovirus in one case.

In our group, no microbial etiology was found, but only 6 out of the 15 patients had that confirmed with chest X-ray. The reason for a small percentage of radiological confirmation was the desire to avoid X-ray exposure and the problem with proper isolation of infectious patients. In some cases, data relating to the onset of varicella infection and respiratory symptoms, or fever were insufficient. In general, the diagnosis was based on clinical presentation, laboratory tests, including blood gases, and chest X-ray whenever possible. The course of pneumonia was generally mild, despite oxygen therapy in two cases.

In the literature, no data exist on other respiratory complications (e.g., bronchitis) as sequelae of varicella infection. The present study demonstrates that, besides pneumonia, some of the children (8/237; 3.4 %, CI: 1.5–6.5 %) developed bronchitis in the course of varicella. We defined cough and bronchial breath sounds as bronchitis, although other than varicella viruses could underlie these symptoms – no advanced lab tests were performed. Albert (2010) and Metlay et al. (1997) draw attention to the difficulties in diagnosing bronchitis and differentiating it from other respiratory diseases, mainly pneumonia.

Since there is no fixed definition of bronchitis, it possibly may be over diagnosed.

In the present study, there was only one immune-compromised girl, suffering from juvenile arthritis treated with methotrexate, who was not vaccinated against varicella. Such children are at increased risk of varicella complications. Another group of children easily exposed to varicella-related complications are those younger than 1 year (Atkinson et al. 2012). We confirmed that as we found that children aged less than 1 were the predominating group with respiratory complications (9/28; 32.1 %). According to the guidelines, children younger than 9 months cannot be vaccinated against varicella nor do they need the application of varicella zoster immunoglobulin (Marin et al. 2007). The situation is additionally complicated by the fact that these children are most often infected with varicella from older siblings being sick; in the present study 8 of the 28 children acquired the infection that way. The literature reports that children infected with varicella by siblings usually have a more negative experience of the disease (Ross et al. 1962). These data let us conclude that the best prophylaxis against varicella infection in infants younger than 1 year is the so-called 'cocoon strategy' – immunization of older siblings who attend school or kindergarten.

In conclusion, despite all the inherent limitations of a retrospective study and, at times, limited availability of detailed microbiological or radiological work-up, we believe we have shown that varicella-related respiratory complications are all too often sequelae of varicella infection and may run a severe course, particularly in children younger than 1 year. We also identified acute bronchitis as a complication of varicella, which has not yet been fully recognized. The findings also point to older siblings in the family as the most probable case of infection spread onto infants.

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

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Hyponatremia in Children Hospitalized due to Pneumonia

16

A. Wrotek and T. Jackowska

Abstract

The aim of this study was to evaluate the relation between hyponatremia (HN) and severity of community-acquired pneumonia (CAP) in children. The study consisted of a retrospective analysis of medical records of 312 children (165 boys, 147 girls) aged 33 days to 16 years, hospitalized with CAP. The children were divided into two age-groups: under and over the age of four. Clinical findings such as breath frequency, heart rate, capillary blood saturation, body temperature, time for defeverscence, duration of antibiotic treatment and hospital stay, and the serum inflammatory markers WBC, neutrophil count, CRP, and procalcitonin level were used as the disease severity predictors. The results demonstrate that hyponatremia was observed in 104/312 (33.3 %) patients. Children with HN of both age-groups had higher neutrophil counts (6.96 vs. $5.73*10^3/\mu L$; $p < 0.05$ and 12.46 vs. $8.22*10^3/\mu L$; $p = 0.01$), those aged > 4 had higher WBC (15.85 vs. $11.0*10^3/\mu L$; $p = 0.02$), and those aged < 4 had a lower lymphocyte count (3.74 vs. $4.75*10^3/\mu L$; $p = 0.02$) than children without HN. Hyponatremic children had higher CRP (28.82 mg/L vs. 9.18 mg/L; $p < 0.01$) and tended to have higher procalcitonin (0.31 vs. 0.19 ng/mL) than children without HN. Body temperature was higher (38.6 vs. 37.6 °C; $p < 0.01$) and duration of hospitalization was longer (9 vs. 8 days, $p = 0.01$) in hyponatremic compared with non-hyponatremic children. There was no correlation between the sodium level and either breath frequency, heart rate, capillary blood saturation, time for defeverscence, or time of antibiotic treatment. We conclude that hyponatremia is a frequent finding in CAP and seems associated with the disease severity.

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_16,

Keywords
Children • Community-acquired pneumonia • Hyponatremia • Hospital stay • Inflammatory markers

1 Introduction

Community-acquired pneumonia (CAP) is a leading single cause of mortality in children under 5 years of age and one of the major causes of hospitalization in children (Rudan et al. 2008). Hyponatremia (HN) is seen in approximately 3 % of hospitalized patients and thus is one of the most frequent electrolyte disorders (Don et al. 2008). Although the first report suggesting the association between pneumonia and hyponatremia was published 40 years ago (Stromont and Waterhouse 1962), there is still not enough data supporting this correlation. HN is seen in 27 % (Singhi and Dhawan 1992) to 45 % (Don et al. 2008) of children hospitalized due to pneumonia. In most cases, HN found in CAP is mild (Don et al. 2008; Sakellaropoulou et al. 2010). It has been suggested that HN is related with severity of pneumonia measured with serum inflammatory markers (Don et al. 2008; Sakellaropoulou et al. 2010), higher body temperature on admission (Don et al. 2008), increased heart rate, tachypnea, longer hospital stay (Sakellaropoulou et al. 2010), or with frequency of complications, higher mortality and longer duration of hospital stay (Singhi and Dhawan 1992). Any correlation between HN and type of radiological consolidation, or etiology of CAP has not been found (Don et al. 2008). The aim of this retrospective analysis was to evaluate the occurrence of HN and to assess the relation between hyponatremia and the severity of community-acquired pneumonia.

2 Methods

The protocol of the study was approved by a local Ethics Committee. This retrospective analysis was conducted in the Department of Pediatrics, the Medical Center of Postgraduate Education and Bielanski Hospital in Warsaw, Poland. The medical files of all the children hospitalized due to pneumonia between January 2009 and December 2010 (24 months) were analyzed. In total, there were 441 patients hospitalized due to pneumonia. Only were children with radiologically confirmed pneumonia eligible for the study. To ascertain the presence of community-acquired, and not nosocomial pneumonia, the children with pneumonia diagnosed within the first 48 h after admission were included. Children with respiratory system and musculoskeletal defects or previous medical interventions which could have an influence on CAP course or facilitate infection (e.g., tetraplegia, lung decortication) were excluded. Exclusion criteria also included previously diagnosed proliferative disease, diabetes mellitus, heart, renal, thyroidal, hypophyseal or adrenal insufficiency and medications altering sodium levels (diuretics). Moreover, children who lacked sodium level measurement on admission also were excluded.

During 24 months, 4,991 patients were hospitalized and 441 (8.8 %) were diagnosed with pneumonia. Twenty eight (6.3 %) children did not meet inclusion criteria and had to be excluded from further analysis. Chest radiographs were taken in 405 of 413 (98.0 %) patients who met the inclusion criteria. CAP was confirmed in 331 (80.1 %) cases. Nineteen other children were excluded from further analysis due to the lack of sodium level measurement. Thus, the final number of the enrolled patients was 312 (165 boys, 147 girls) aged between 33 days and 16 years (Fig. 16.1).

HN was defined as a serum sodium concentration under 136 mmol/L and was stratified into mild (131–135 mmol/L), moderate (126–130 mmol/L), and severe (125 mmol/L and lower) (Ellison and

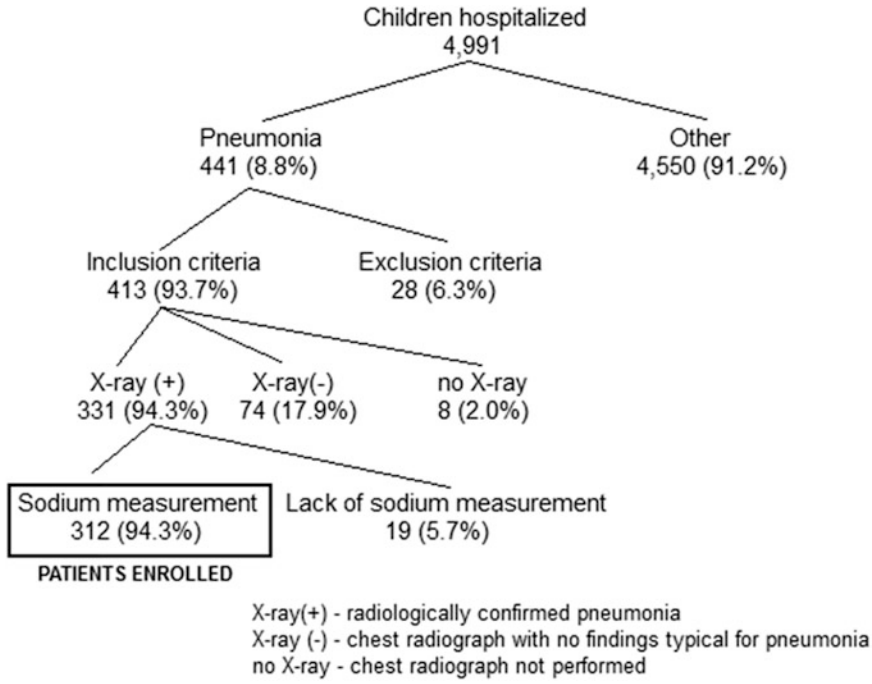

Fig. 16.1 Structure of hospitalizations 2009–2010 and patients enrolled

Berl 2007). Since there is no widely-accepted and evidence-based scale for rating severity of pneumonia in children, we used clinical findings (breath frequency, heart rate, capillary blood saturation, body temperature, time for defeverscence, duration of antibiotic treatment, and duration of hospital stay), and serum inflammatory markers (white blood cells count, neutrophil count, serum C-reactive protein, and procalcitonin) as disease severity predictors. Special attention was paid to physiological differences in age groups, so breath frequency was analyzed separately in infants and in children aged one and more, also white blood cell count and neutrophil/lymphocyte count were analyzed separately for children under 4 years and older.

Shapiro-Wilk test was used to determine whether data was distributed normally. For normally distributed data, the independent-samples *t*-test was used. The Mann–Whitney U test was used for variables that were not distributed normally. $P < 0.05$ was considered as statistically significant. Statistical analysis was performed using Statistica 10 software.

3 Results

Hyponatremia was observed in 104 of 312 (33.3 %) children. Sodium levels ranged from 126 to 146 mmol/L. HN was mild in 100 (96.2 %) and moderate in four children, with no cases of severe HN. Body temperature on admission was enhanced (38.6 °C vs. 37.6 °C; $p < 0.01$) and duration of hospital stay was longer (9 days vs. 8 days, $p = 0.01$) in the hyponatremic children compared with the children without HN. There was no correlation between the sodium levels and either breath frequency, heart rate, capillary blood saturation, time for defeverscence, or time of antibiotic treatment (Table 16.1).

On admission, children with HN had higher neutrophil count in both groups, under and over 4 years of age (medians $6.96*10^3$/μL vs. $5.73*10^3$/μL ($p < 0.05$) and $12.46*10^3$/μL vs. $8.22*10^3$/μL ($p = 0.01$), respectively) than those without HN. In the hyponatremic children, a higher total white blood cells count (WBC) in the group over 4 years of age $15.85*10^3$/μL vs. $11*10^3$/μL ($p = 0.02$) and a lower lymphocyte count in the group under

Table 16.1 Clinical data of hyponatremic (HN) and normonatremic children

	Children with HN			Children without HN			
	Median	Pc 25	Pc 75	Median	Pc 25	Pc 75	p
Fever (°C)	38.6	37.4	39.2	37.6	36.8	38.8	0.001
Breath frequency (per min)	30.0	20.0	40.0	31.0	24.0	50.0	0.238
Heart rate (per min)	116.0	100.0	120.0	120.0	100.0	130.0	0.377
Capillary blood oxygen saturation (%)	91.0	87.5	95.5	94.0	91.0	96.0	0.257
Defeverscence (h)	36.0	24.0	60.0	48.0	36.0	60.0	0.754
Antibiotic treatment (days)	10.0	9.5	11.0	10.0	9.0	10.5	0.279
Hospitalization (days)	9.0	6.0	11.0	8.0	6.0	10.0	0.011

Percentile (Pc) 25; Percentile (Pc) 75

4 years of age $3.74*10^3/\mu L$ vs. $4.75*10^3/\mu L$ ($p = 0.02$) were observed than the respective values in the children without HN. The hyponatremic children also had a higher serum C-reactive protein, median 28.82 mg/L vs. 9.18 mg/L ($p < 0.01$), and procalcitonin levels (0.31 ng/mL vs. 0.19 ng/mL) ($p = 0.054$) than the respective values in the children without HN. Other laboratory results did not show appreciable differences, i.e., WBC in the children under 4 years of age or lymphocyte count in the children of under 4 years of age and older (Table 16.2).

4 Discussion

The results of our study confirm that hyponatremia is a frequent finding in children with CAP and may be related to disease's severity, which is in agreement with the few existing studies on the subject. We found HN in 33.3 % cases of CAP. Sakellaropoulou et al. (2010) determined HN to occur in 35.2 % of children with CAP, while Don et al. (2008) found 45 % of patients with pneumonia to be hyponatremic. In both studies, the prevalence of mild forms of HN (96 and 92 %, respectively) is in line with our result of the 96 %. The percentage of moderate and severe HN published by Singhi and Dhawan (1992) was higher (27 and 4.5 %, respectively). Yet the higher percentage may result from a different severity of pneumonia in that study in which 13 out of the 264 patients (5 %) with moderate-to-severe HN died.

In previous studies, severity of pneumonia was assessed by laboratory findings such as serum inflammatory markers (CRP, PCT, WBC, or ESR). These markers were enhanced in hyponatremic children (Don et al. 2008; Sakellaropoulou et al. 2010). Our study confirmed that the presence of HN was associated with increased CRP concentrations, WBC count (in children over 4 years of age), and neutrophil count, while the association with PCT remains to be further analyzed. The present study is limited by a small number of PCT measurements (only in 52 out of the 312 patients, 16.7 %). Clinical features (such as fever, time for defeverscence, tachypnea, increased heart rate, duration of hospital stay, and antibiotic treatment) have also been used to assess the severity of pneumonia. In a study of Don et al. (2008) children with HN had higher body temperature on admission, increased heart rate, tachypnea, and were hospitalized for a longer time. In our present study, children with HN also presented higher body temperature on admission and required longer hospitalization; yet there was no correlation between HN and duration of antibiotic treatment. The median time of antibiotic treatment was longer than that of hospitalization since treatment continued after discharge on an outpatient basis.

According to Singhi and Dhawan (1992), there is a correlation between HN and a higher rate of complications, higher mortality, and longer duration of hospitalization. However, in that study only were patients with moderate-to-severe HN analyzed, so the groups are different. In our study, because of a small number of patients who had complications and unfavorable outcome, statistical analysis concerning association of HN

Table 16.2 Laboratory data of hyponatremic (HN) and normonatremic children

	Children with HN			Children without HN			
	Median value	Pc 25	Pc 75	Median value	Pc 25	Pc 75	p
All ages							
WBC (*10^3/μL)	13.90	9.40	20.00	12.30	9.40	16.50	0.078
Neu (*10^3/μL)	7.65	4.20	14.05	6.03	3.08	10.80	0.002
Lym (*10^3/μL)	3.37	2.18	5.01	4.42	2.80	6.36	0.001
CRP (mg/L)	28.82	6.12	111.46	9.18	1.40	27.96	0.001
PCT (ng/mL)	0.31	0.14	2.13	0.19	0.10	0.42	0.054
Under 4 years old							
WBC (*10^3/μL)	13.10	9.10	19.00	12.40	9.60	16.40	0.465
Neu (*10^3/μL)	6.96	4.14	13.14	5.73	3.04	10.41	0.048
Lym (*10^3/μL)	3.74	2.57	5.96	4.75	3.22	6.68	0.020
Over 4 years old							
WBC (*10^3/μL)	15.85	9.80	25.40	11.00	6.30	16.85	0.024
Neu (*10^3/μL)	12.46	6.09	21.14	8.22	3.48	11.55	0.010
Lym (*10^3/μL)	2.29	1.80	3.22	2.24	1.85	3.70	0.830

Percentile (Pc) 25; Percentile (Pc) 75

with frequency of complications or mortality could not be conclusively carried out.

Hyponatremia may be caused by free water retention, sodium shift (from extracellular to intracellular compartment), water shift (from intracellular to extracellular compartment), or simply by excessive hypotonic fluid intake. Singhi and Dhawan (1992) suggested that a syndrome of inappropriate secretion of antidiuretic hormone (SIADH) may be the underlying mechanism of pneumonia-related HN. Antidiuretic syndrome (ADH, vasopressin) leads to water retention, which results in plasma dilution while the amount of extracellular sodium is not decreased. Therefore, excessive fluid intake may lead to a further decrease in sodium concentrations. There were no clinical trials in children that would show increased vasopressin concentrations in patients with SIADH, which may be due likely to difficulties in the measurement of circulating vasopressin. The hormone is unstable (half-life time ranges from 5 to 15 min) and attached to platelets (Muller et al. 2007). Copeptin, which is co-synthesized with vasopressin reflects vasopressin concentrations and is more stable than ADH. The study concentrating on copeptin levels in the course of pneumonia could show a role of SIADH.

The influence of iatrogenic hyponatremia on the outcome of CAP remains unknown, although Horn et al. (2004) reported that children who receive significantly more intravenous fluids during hospitalization develop hyponatremia; two of those patients had major neurologic sequelae and one died. Therefore, the authors recommend that hypotonic fluid should not be given to children with sodium levels under 138 mmol/L. Halberthal et al. (2001) suggested the use of hypotonic fluid only in children with sodium levels greater than 140 mmol/L, which emphasizes the danger of routine use of hypotonic fluids.

In conclusion, our results confirmed that mild hyponatremia is a frequent finding in CAP and seems to be associated with disease severity as assessed by non-specific inflammatory markers, fever, and time for hospitalization. Further studies on the effects of sodium levels on clinical course of CAP and on the pathophysiological pathway of CAP-related hyponatremia are required.

Acknowledgments Supported by grant 502-1-20-01-12 of the Medical Center of Postgraduate Education in Warsaw.

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

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17 *In Vitro* Sensitivity of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* to Carbapenems Among Intensive Care Unit Patients

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Abstract

Acinetobacter baumannii and *Pseudomonas aeruginosa* pathogens are the most common causes of fatal pneumonia among patients treated in Intensive Care Units (ICU). Carbapenems remain a group of antibiotics characterized by the highest effectiveness in treatment of heavy infections of the lower respiratory tract. This study compared *in vitro* sensitivity of *A. baumannii* and *P. aeruginosa* to three carbapenems: imipenem, meropenem and doripenem. The material was collected from 71 patients treated in the ICU from April 2009 to January 2010. Bronchial tree was the predominant source of samples. Fifty-four strains of *A. baumannii* and 17 strains of *P. aeruginosa* were analyzed. Sensitivity to carbapenems was interpreted in line with Clinical and Laboratory Standard Institute (CLSI) and European Committee for Antimicrobial Susceptibility Testing (EUCAST) criteria (imipenem and meropenem) or in compliance with the Food and Drug Administration (FDA) and CLSI guidelines (doripenem). We found that *A. baumannii* was significantly more often sensitive to imipenem than to doripenem and meropenem, but only according to the CLSI and FDA and not EUCAST criteria. The sensitivity of *P. aeruginosa* was higher to imipenem than to doripenem and meropenem, according to both CLSI and EUCAST criteria (64.7 %).

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_17,

We conclude that the EUCAST criteria demonstrate a higher rigor than those of CLSI and FDA in the determination of carbapenems sensitivity. Imipenem appears more effective than doripenem and meropenem in treatment of *A. baumannii* and *P. aeruginosa* infections.

Keywords
Acinetobacter baumannii • Carbapenems • *Pseudomonas aeruginosa* • Resistance • Sensitivity

1 Introduction

Infections, primarily due to pneumonia, account for the major cause of fatalities occurring in Intensive Care Units (ICU). Ventilator-associated pneumonia (VAP) occurs in 10–20 % of patients mechanically ventilated for more than 48 h and the mortality rate resulting from pneumonia is at a high level and ranges from 30 to 70 % (Kollef et al. 2005). High percentages of therapy failures in patients with infections treated in ICU are associated with improper choice of antibiotics for empiric therapy, whose spectrum does not cover the wide range of microorganisms and their mechanism of resistance. Empiric antibiotic therapy should follow the generally accepted current recommendations and consider the information about local pathogens and their susceptibility. The effectiveness of individual therapy regimens differs according to the country, region, and health institution, and depends markedly on the spectrum of microorganisms, antibiotics used earlier and the resultant mechanism of resistance (Koziol-Montewska et al. 2011).

Antibiotic therapy in ICU, including antibiotic therapy for VAP, should be a compromise between appropriate and early pharmacotherapy based on broad-spectrum antibiotics, knowledge of local epidemiology and susceptible bacteria, and fear of increasing resistance to the antibiotics presently available (Koziol-Montewska et al. 2011; Magnotti et al. 2008). Both delayed treatment and improper therapy (an antibiotic ineffective against infections producing pathogens, improper dose or pharmacodynamic parameters, and therapy duration) are associated with worse outcomes. Thus, surveillance of microorganisms responsible for infections in ICU should be strongly recommended and the microbiological pattern, including the susceptibility and resistance patterns of cultured bacteria, should be conducted and systematically repeated and analyzed in each ICU.

The common causes of infection in ICU are *Pseudomonas aeruginosa* and *Acinetobacter baumannii* pathogens (Koziol-Montewska et al. 2011; Magnotti et al. 2008; Kollef et al. 2005). In a prevalence study of infections in intensive care units conducted among 75 countries of the 5 continents, *Acinetobacter baumannii* has been found to be the fifth most common pathogen, although with a high variability among different countries (Vincent et al. 2009). Different surveillance studies have found this pathogen to be the fifth cause of pneumonia, after *P. aeruginosa*, in hospitalized patients, mainly in ICU (Jones 2010). In addition, these microorganisms are also frequently reported to cause other nosocomial infections such as bacteremia and urinary tract and surgical infections. In fact, *A. baumannii* was found to be the third most frequent cause of nosocomial bloodstream infection in a large multicenter study with an estimation of 34 % of all patients and 43 % in patients in ICU (Wisplinghoff et al. 2004). With respect to the treatment of *A. baumannii* infections, it is important to take into account the resistance profile involved to consider the different treatment options available.

A. baumannii has become resistant to almost all commonly used antimicrobial agents, including aminoglicosides, quinolones, and broad-

spectrum betalactamases, and multidrug or pandrug resistance strains, including strains resistant to carbapenems and occasionally colistin, have appeared (Rossolin and Mantegnoli 2008; Livermore et al. 2008). Data from many European centers show an increasing resistance of *P. aeruginosa* strains to carbapanems, conditioned mainly by beta-lactamase synthesis. In a tertiary hospital in Lithuania, the prevalence of carbapenem-resistant *P. aeruginosa* strains increased from 10 to 40 % (Vitkauskiene et al. 2011). Increasing resistance to carbapenems is a cause of concern because of nosocomial infections and it adversely affects clinical outcomes and adds to treatment costs.

The aim of the present study was to evaluate the effectiveness of three antibiotics of the carbapenems class, i.e., doripenem, imipenem and meropenem on the basis of *in vitro* sensitivity in compliance with the criteria of the Clinical and Laboratory Standards Institute (2008) and European Committee for Antimicrobial Susceptibility Testing (EUCAST) (2011) classification in case of imipenem and meropenem and of the Food and Drug Administration (FDA) and EUCAST in case of doripenem.

2 Methods

2.1 Collection and Processing of Biological Samples

The study was approved by the Ethics Committee of the Institute of Military Medicine in Warsaw, Poland. Fifty four strains of *A. baumannii*: 47 (87.0 %) from tracheal swabs, 3 (5.6 %) from wound swabs, 2 (3.7 %) from blood culture, 1 (1.9 %) from bronchoalveolar lavage, and 1 (1.9 %) from a drain site, and 17 strains of *P. aeruginosa*: 16 (94.1 %) from tracheal swabs and 1 (5.9 %) from pleural fluid culture, were subjected to analysis. The material was collected from 71 patients treated in the Clinic of Anesthesiology and Intensive Care of Institute of Military Medicine in Warsaw, Poland. All of the strains were isolated in the period from April 2009 to January 2010.

The isolated material was identified in an automated microbiology system VITEK 2 (bioMérieux) by means of GN cards, following the guidelines issued by manufacturer. The strains which had been identified were subjected to manual determination of Minimal Inhibitory Concentration (MIC) value by means of Etest® bioMérieux gradient strips which measure the concentration of a given antibiotic (the analyzed concentration range for imipenem 0.002–32 mg/L, meropenem 0.002–32 mg/L, doripenem 0.002–32 mg/L) on the Müeller-Hinton plates (bioMérieux, France). The MIC limit values, meant for classifying the strain as sensitive or resistant according to the American CLSI and FDA criteria (Food and Drug Administration 2011; Clinical and Laboratory Standards Institute 2008) and European EUCAST guidelines (The European Committee on Antimicrobial Testing. EUCAST criteria 2011) were presented in Table 17.1.

The next stage of the study was a genetic analysis conducted in the Institute of Molecular Microbiology of the National Medicines Institute in Warsaw, Poland. Owing to predominance of *A. baumannii* isolates, 10 randomly selected strains of this particular isolate became subject to molecular genotyping (*P. aeruginosa* isolates were not subject to typing) by means of a referential method based on *Restriction Fragment Length Polymorphism* (restriction enzyme Apal) of the genome using the procedure of *Pulsed-Field Gel Electrophoresis* (RFLP-PFGE) (Fig. 17.1).

2.2 Statistical Analysis

Chi^2 test was applied to calculate statistical drug sensitivity of bacterial strains studied with respect to particular carbapenems and classification standards. A $p < 0.05$ was determined as

Table 17.1 MIC values of *A. baumannii* and *P. aeruginosa* strains to carbapenems according to CLSI (imipenem, meropenem), FDA (doripenem) and EUCAST (imipenem, meropenem, doripenem) classification

	Imipenem mg/L		Meropenem mg/L		Doripenem mg/L	
Bacterial strain	Sensitivity	Resistance	Sensitivity	Resistance	Sensitivity	Resistance
MIC values according to CLSI and FDA classifications						
Acinetobacter baumannii	≤4	≥16	≤4	≥16	≤1	>1
Pseudomonas aeruginosa	≤4	≥16	≤4	≥16	≤2	>2
MIC values according to EUCAST classification						
Acinetobacter baumannii	≤2	>8	≤2	>8	≤1	>4
Pseudomonas aeruginosa	≤4	>8	≤2	>8	≤1	>4

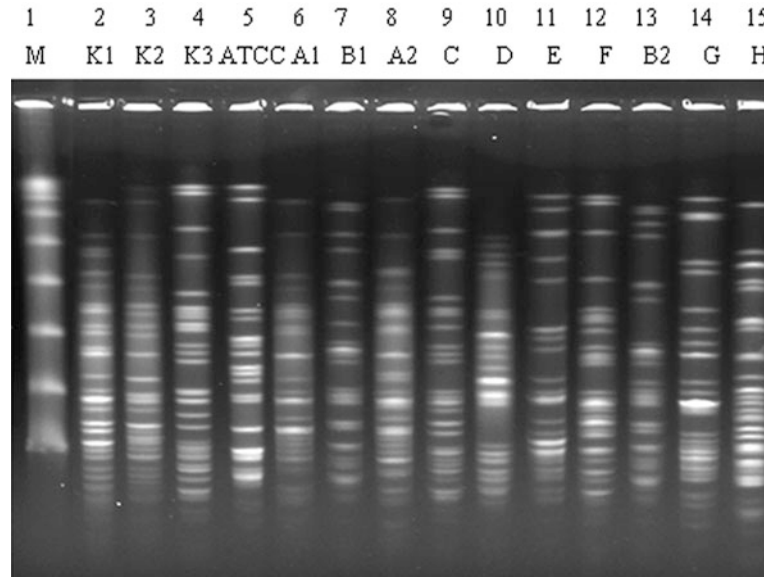

Fig. 17.1 PFGE of ApaI restriction of representative *A. baumannii* isolates, demonstrating PFGE type A (A1, A2), type B (B1, B2), patterns C, D, E, F, G, H. Also in this figure, M – DNA lambda ladder PFGE marker (New England BioLabs, USA); K1,K2,K3 – controls; ATCC – isolate marker *A. baumannii* ATCC 19606

significant. Calculations were performed using program Statistica PL (license number SN: SP 7105488009 G51).

3 Results

As a result of the genetic study conducted, 10 patterns of electrophoretic separations were identified for *A. baumannii*. Proximity analysis of the electrophoretic patterns revealed 2 pairs of mutually related isolates: type A (subtype A1, A2) and type B (subtype B1 and B2). The remaining isolates demonstrated unique separation patterns C, D, E, F, G, and H.

Table 17.2 demonstrates sensitivity of *A. baumannii* and *P. aeruginosa* strains to carbapenems according to the American and European criteria and Table 17.3 illustrates the comparison of *A. baumannii* and *P. aeruginosa* sensitivity to carbapenems. The percentage of *A. baumannii* strains sensitive to doripenem following the guidelines of both classifications was identical – 37.0 %, whereas the proportion of sensitive *P. aeruginosa* strains differed between both classifications – 64.7 % according to the FDA and 47.1 % according to the EUCAST criteria (Table 17.2).

The percentage of *A. baumannii* strains sensitive to imipenem according to the CLSI criteria was higher than that in case of the EUCAST classification – 68.5 % vs. 50.0 %, whereas the percentage of sensitive *P. aeruginosa* strains studied in compliance with both classifications was identical (Table 17.2). The proportion of

Table 17.2 Sensitivity of *A. baumannii* and *P. aeruginosa* strains to doripenem (according to FDA and EUCAST criteria), imipenem and meropenem (according to CLSI and EUCAST criteria)

		FDA	EUCAST
Bacterial strain (*n*)	Sensitivity to doripenem	n (%)	n (%)
Acinetobacter baumannii (*n* = 54)	Sensitive	20 (37.0)	20 (37.0)
	Intermediate	–	20 (37.0)
	Resistant	32 (59.3)	12 (22.2)
	No data	2 (3.7)	2 (3.7)
Pseudomonas aeruginosa (*n* = 17)	Sensitive	11 (64.7)	8 (47.1)
	Intermediate	–	4 (23.5)
	Resistant	5 (29.4)	4 (23.5)
	No data	1 (5.9)	1 (5.9)
		CLSI	EUCAST
Bacterial strain (*n*)	Sensitivity to imipenem	n (%)	n (%)
Acinetobacter baumannii (*n* = 54)	Sensitive	37 (68.5)	27 (50.0)
	Intermediate	6 (11.1)	15 (27.8)
	Resistant	11 (20.4)	12 (22.2)
Pseudomonas aeruginosa (*n* = 17)	Sensitive	11 (64.7)	11 (64.7)
	Resistant	6 (35.3)	6 (35.3)
		CLSI	EUCAST
Bacterial strain (*n*)	Sensitivity to meropenem	n (%)	N (%)
Acinetobacter baumannii (*n* = 54)	Sensitive	29 (53.7)	15 (27.8)
	Intermediate	11 (20.4)	16 (29.6)
	Resistant	14 (25.9)	23 (42.6)
Pseudomonas aeruginosa (*n* = 17)	Sensitive	11 (64.7)	7 (41.2)
	Intermediate	–	4 (23.5)
	Resistant	6 (35.3)	6 (35.3)

n – number of strains

bacterial strains sensitive to meropenem was higher according to the CLSI criteria than that in case of the EUCAST classification – 53.7 % vs. 27.8 % for *A. baumannii* and 64.7 % vs. 41.2 % for *P. aeruginosa* (Table 17.2).

A. baumannii strains demonstrated the highest sensitivity to imipenem according to both classifications (Table 17.3). However, this observation was statistically significant only when the CLSI guidelines were considered. Sensitivity of *P. aeruginosa* to all studied carbapenems assessed both with CLSI and FDA classifications was not appreciably different (Table 17.3).

4 Discussion

Gram-negative bacterial infections, especially those described as multiple drug resistance (MDR) strains pose major epidemiological risk as far as nosocomial infections are concerned. The most common pathogens causing such infections are non-fermenting Gram-negative coccobacilli of the *Pseudomonas* spp. and *Acinetobacter* spp. genus and of the *Enterobacteriaceae* spp. family. The literature shows that a substantial fraction of infections acquired in ICU have been due to Gram-negative bacteria, including *P. aeruginosa* (14.2 %) and *A. baumannii* (15.3 %) (Kübler et al. 2004).

Carbapenems, which were first introduced in the 1980s, proved to be the most effective group of antibiotics, and in many cases they were used as a last resort in treating infections induced by Gram-negative bacteria above outlined. Unfortunately, in recent years carbapenems have appeared less effective in a considerable number of infections. Carbapenem resistance in *Acinetobacter* and

Table 17.3 Comparison of *A. baumannii* and *P. aeruginosa* sensitivity to carbapenems according to CLSI, FDA, and EUCAST criteria

Sensitivity of *A. baumannii* according to CLSI and FDA	Doripenem ($n = 52$)[a] n (%)	Meropenem ($n = 54$) n (%)	Imipenem ($n = 54$) n (%)	p
Sensitive	20 (38.5)	29 (53.7)	37 (68.5)	0.008
Intermediate + resistant	32 (61.5)	25 (46.3)	17 (32.5)	
Sensitivity of *A. baumannii* according to EUCAST	Doripenem ($n = 52$)[a] n (%)	Meropenem ($n = 54$) n (%)	Imipenem ($n = 54$) n (%)	P
Sensitive	20 (38.5)	15 (27..8)	27 (50.0)	0.06
Intermediate + resistant	32 (61.5)	39 (72.2)	27 (50.0)	
Sensitivity of *P. aeruginosa* according to CLSI and FDA	Doripenem ($n = 16$)[b] n (%)	Meropenem ($n = 17$) n (%)	Imipenem ($n = 17$) n (%)	P
Sensitive	11 (68.8)	11 (64.7)	11 (64.7)	0.96
Intermediate + resistant	5 (31.2)	6 (35.3)	6 (35.3)	
Sensitivity of *P. aeruginosa* according to EUCAST	Doripenem ($n = 16$)[b] n (%)	Meropenem ($n = 17$) n (%)	Imipenem ($n = 17$) n (%)	P
Sensitive	8 (50.0)	7 (41.2)	11 (64.7)	0.38
Intermediate + resistant	8 (50.0)	10 (58.8)	6 (35.3)	

[a]In two cases sensitivity to doripenem was not determined
[b]In one case sensitivity to doripenem was not determined

Pseudomonas infections is increasingly observed worldwide and constitutes a sentinel event for emerging antimicrobial resistance (Roca et al. 2012; Poirel and Nordman 2006).

The results of a recent study on bacterial resistance to antibiotics, based on the data collected in 35 ICU in 13 European countries indicate that *A. baumannii* resistance to imipenem ranged from 0 % in Estonia and Sweden, 10–20 % in the majority of other countries, to 38 % in Turkey, and as much as 90 % in Malta. *P. aeruginosa* resistance to the drug was estimated at the level of 13 % in Estonia to 48 % in Turkey. The growth in prevalence of antibiotic resistant bacteria is commonly associated with increased consumption of antibiotics. The lowest consumption, i.e., 426–638 DDD/1,000 beds, was registered in Switzerland; while in other European countries it was approximately 1,254 DDD/1,000 beds. An interesting fact remains that no correlation between the use of antibiotics and a number infections induced by the so-called alert pathogens occurring in ICUs has been observed, since that greatly depends on a constant inflow of patients infected with such pathogens (Hanberger et al. 2009).

There are slight differences in the properties of imipenem, meropenem, and doripenem. The distinction between the first two mentioned is of no clinical significance. However, in case of doripenem, the most recently developed carbapenem effective mainly against Gram-negative bacteria, especially *Pseudomonas aeruginosa*, the difference is quite important. A study which analyzed *in vitro* sensitivity of 6,000 bacteria to imipenem and meropenem demonstrated a lower MIC value for meropenem toward *Enterobacteriaceae* and *Pseudomonas aeruginosa* than that for imipenem. On the other hand, imipenem was characterized by a lower MIC value for *A. baumannii*. A Meropenem Yearly Susceptibility Test Information Collection (MYSTIC) study indicated that meropenem is more effective toward *Acinetobacter* spp., *Pseudomonas* spp. and *Enterobacteriaceae* spp. than imipenem, whereas the latter was more effective toward *E. faecalis* (Hoban et al. 1993).

A major multicenter study on the usefulness of doripenem in treating hospital-acquired pneumonia (HAP) demonstrated that its effectiveness is similar to that of imipenem 68.3 vs. 64.2 %; whereas regarding *P. aeruginosa* it was higher – 80.0 vs. 42.9 %, respectively, although that result

failed to be of statistical significance (Chastre et al. 2008). The above mentioned results apply to bacteria cultivated from samples collected in ICUs, which means bacterial strains of the highest possible resistance. Genetic studies of *Acinetobacter* strains demonstrated a diversity of electrophoretic separations; confirming that the subject for analysis was not a single, separate type of bacteria.

In the present study, sensitivity of the bacterial strains was interpreted in compliance with two separate criteria, the American and European criteria. That, in our opinion, contributes to clinical importance of the study. Adopting the EUCAST criteria, more rigorous regarding the category of sensitivity, seems justifiable in the assessment of antibiotic treatment in ICUs. This particularly applies to sensitivity of *P. aeruginosa* to doripenem, *P. aeruginosa*, and *A. baumannii* to meropenem, and also sensitivity of *A. baumannii* to imipenem. Our data showed that in both classifications *A. baumannii* demonstrated a higher sensitivity to imipenem; however, statistical significance only applied to the CLSI guidelines. No differences regarding the sensitivity of *P. aeruginosa* to the three carbapenems studied emerged. Also, the finding of other authors (Pillar et al. 2008) of higher *in vitro* sensitivity of doripenem than that that of imipenem and meropenem toward *P. aeruginosa* was not confirmed.

The present study shows that, considering an *in vitro* assessment, carbapenems remain an effective group of antibiotics as far as treating *P. aeruginosa* and *A. baumannii* infection is concerned. This result is consistent with the observation of Koziol-Montewska et al. (2011) that in a pool of VAP pathogens, carbapenems (imipenem, meropenem, doripenem) seem to be the drugs for empiric antibiotic therapy. The question why imipenem, which is the oldest carbapenem, demonstrates a better effectiveness toward the bacteria analyzed than the two remaining carbapenems cannot be answered unequivocally. From the standpoint of pharmacotherapy, one of the better properties of imipenem is its ability to bind with penicillin binding proteins (PBP-2) of bacteria regardless of concentration, whereas in case of meropenem such combination is only possible in higher concentrations (Hanberger et al. 2009).

It should be underlined that multidrug resistant/carbapenem resistant strains of *A. baumannii* and *P. aeruginosa* are associated with treatment challenges, which emphasize the importance of preventing and controlling the dissemination of the strains (Poirel and Nordman 2006). Infection control measures, such as culture surveillance with recognition of susceptibility and resistance patterns, contact precautions, cohorts, source identification, and environmental control should all be introduced to prevent dissemination of multidrug resistant microorganisms (Romanelli et al. 2009; Urban et al. 2003). Hospitals, mainly ICUs, should introduce the antibiotic policy based on permission system to control the use of carbapenems which can efficiently suppress the incidence of drug resistance bacteria (Ikeda et al. 2012).

5 Conclusions

The EUCAST criteria, as opposed to the CLSI and FDA guidelines, demonstrate a higher rigor regarding the category of sensitivity for carbapenems that remain an effective group of antibiotics in treatment of *A. baumannii* and *P. aeruginosa* infections. Imipenem was confirmed *in vitro* to be more effective an antibiotic in comparison with doripenem and meropenem in such infections.

Acknowledgments We thank Dr. Dariusz Tomaszewski from the Clinic of Anesthesiology and Intensive Care of the Military Institute of Medicine in Warsaw for stylistic editing of the manuscript.

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

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Prevalence of Acute Respiratory Tract Diseases Among Soldiers Deployed for Military Operations in Iraq and Afghanistan

18

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Abstract

Respiratory diseases are one of the most common health problems among service personnel assigned to contemporary military operations which are conducted in areas characterized by adverse environmental conditions. This article reviews the results of the studies into the prevalence of acute respiratory tract diseases among soldiers of the Polish Military Contingent deployed to Iraq and Afghanistan. The article also discusses a number of factors which increase the prevalence of diseases diagnosed in the population of soldiers on a military mission in different climatic and sanitary conditions. Retrospective analysis was based on medical records of Polish troops treated on an outpatient basis in Iraq in 2003–2004 (n = 871) and in Afghanistan in 2003–2005 (n = 400), 2009 (n = 2,300), and 2010 (n = 2,500). The intensity rates were calculated and were then used to calculate the prevalence of diseases per 100 persons in a given population of the military personnel. We found that acute respiratory tract diseases were one of the most common health problems treated in outpatient medical facilities in all four study populations. The incidence rate was 45.6 cases in Iraq in 2003–2004, and in Afghanistan it amounted to 61.8 in 2003–2005, 45.3 in 2009, and 54.8–100 persons in 2010. In conclusion, the prevalence of respiratory diseases was closely related to the

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_18,

environmental factors, such as sand and dust storms, extreme temperature changes, unsatisfactory sanitary conditions, and common disregard of basic principles concerning disease prevention.

Keywords
Disease prevention • Military medical service • Prevalence • Respiratory tract diseases • Soldiers

1 Introduction

Military operations which were launched in Iraq or Afghanistan are carried out in areas in which difficult environmental conditions prevail. As a result of ongoing hostilities, there is an increased risk of various diseases and injuries among the local population, and in the population of military personnel serving in the international coalition forces. The factors which are responsible for increased prevalence of infectious and non-infectious diseases in the population of soldiers participating in contemporary combat operations (Soltis et al. 2009; Aronson et al. 2006) include adverse environmental conditions, such as desert climate in Iraq, high mountain climate in Afghanistan, sand and dust storms, extreme temperature changes within 24 h, unsatisfactory sanitary conditions, and common disregard of basic principles concerning disease prevention. The most common health problems reported by military personnel who are treated in outpatient medical facilities, apart from traumatic profile associated with battle and non-battle injuries, include respiratory, gastrointestinal, and skin diseases, with the predominance of upper airway infections.

The aim of this article was to present the results of search into the prevalence of respiratory tract diseases among soldiers of Polish military contingents deployed to Iraq and Afghanistan. A number of different factors which increase the prevalence of diseases diagnosed in the population of soldiers on a military mission in different climatic and sanitary conditions were also taken into account.

2 Methods

The study was approved by the Ethics Committee of the Military Institute of Medicine in Warsaw, Poland.

2.1 Data Collection

The analysis of acute respiratory tract diseases diagnosed in the members of Polish Military Contingents relocated to Iraq and Afghanistan was based on medical records and laboratory tests results of soldiers treated on an outpatient basis (initial visits, excluding check-up appointments) in military health care facilities. The study population was divided into four groups:

- sick calls in the Polish Field Hospital in Iraq in the period 2003–2004 – 871 soldiers stationing in two military bases: Camp Juliet and Camp Lima in Karbala Province;
- outpatient medical facility of the Polish Military Contingent in Afghanistan in the period 2003–2005 – 400 soldiers stationing in Bagram Airfield in the Parwan Province;
- sick calls in the Polish Field Hospital in Afghanistan in 2009–2300 soldiers stationing in Forward Operating Base Ghazni in the Ghazni Province;
- sick calls in the Polish Field Hospital in Afghanistan in 2010–2500 soldiers stationing in Forward Operating Base Ghazni in the Ghazni Province.

A retrospective study made it possible to estimate the intensity rate which was used to calculate the prevalence of cases of diseases per 100

persons (see below). The four study populations were of random composition. Changes in the confidence level of $p < 0.05$ were assumed to be relevant. The data collected, were then presented in the form of figures.

2.2 Case Definitions

Diseases affecting particular organs and systems were diagnosed in accord with the ICD-9 CM classification: respiratory, circulatory, gastrointestinal, musculoskeletal, neurological, urogenital, eye, ear diseases, contagious and parasitic diseases, mental disorders, and injuries. Detailed diagnoses of specific disease entities were interpreted in compliance with the same classification.

2.3 Statistics

The basis for calculating the intensity rate was the number of admissions according to diagnosed diseases used as a numerator divided by the total number of people in the study population within the analyzed period as a denominator ($n = 871$ soldiers of PMC Iraq; $n = 400$ soldiers of PMC Afghanistan in years 2003–2005, $n = 2{,}300$ in 2009, $n = 2{,}500$ in 2010) multiplied by the coefficient $C = 10^k$ ($k = 0,1,2,3\ldots$, in the statistical analysis $k = 2$), which was used to calculate the prevalence of cases of diseases per 100 persons in the study population. STATISTICA PL software was used to calculate the final scores.

3 Results

The research demonstrated that respiratory tract diseases were the most common health problem treated on an outpatient basis in medical facilities supporting Polish Military Contingents in Iraq and Afghanistan in all four study populations. The prevalent health problems reported among 871 Polish military personnel treated in the sick call of the Polish Field Hospital in Iraq in years 2003–2004 were respiratory tract diseases (45.6 cases per 100 persons), dermatoses, injuries, and gastrointestinal diseases (Fig. 18.1).

Respiratory illnesses diagnosed in the group of 871 soldiers serving in Iraq included 397 cases of acute upper respiratory tract diseases: cold (26.9/100 persons), pharyngitis and/or tonsillitis (13.3/100 persons), sinusitis (3.1/100 persons), and bronchitis (2.3/100 persons). There was an increased prevalence of diseases in March-April and again in September-October, that is, when the Polish contingent rotated its troops and when the newly-arriving soldiers were undergoing the acclimatization process to adjust to environmental conditions prevailing in the theater of operations.

The most common health problems reported among 400 soldiers treated in the outpatient medical facility of the Polish Military Contingent in Afghanistan in the years 2003–2005 were respiratory diseases (61.8 cases/100 persons), dermatoses, injuries, and gastrointestinal diseases (Fig. 18.2).

Two hundred forty seven cases of respiratory tract illnesses were diagnosed among 400 soldiers serving in Afghanistan. The sickness consisted of cold (37.8/100 persons), pharyngitis and/or tonsillitis (17.0/100 persons), sinusitis (4.3/100 persons), bronchitis (1.0/100 persons), pneumonia (0.5/100 persons), and others (1.2/100 persons).

The prevalent health problems reported among 2,300 Polish military personnel treated in the sick call of the Polish Field Hospital in Afghanistan in 2009 were respiratory diseases (45.3 cases per 100 persons), injuries, gastrointestinal, and skin diseases (Fig. 18.3).

One thousand forty three cases of respiratory tract illnesses were diagnosed in the group of 2,300 soldiers relocated to Afghanistan in 2009. The sickness consisted of cold (31.0/100 persons), pharyngitis and/or tonsillitis (11.2/100 persons), sinusitis (2.0/100 persons), bronchitis (1.0/100 persons), and pneumonia (0.1/100 persons).

The most common health problems reported among 2,500 Polish military personnel treated in the sick call of the Polish Field Hospital in Afghanistan in 2010 were respiratory diseases

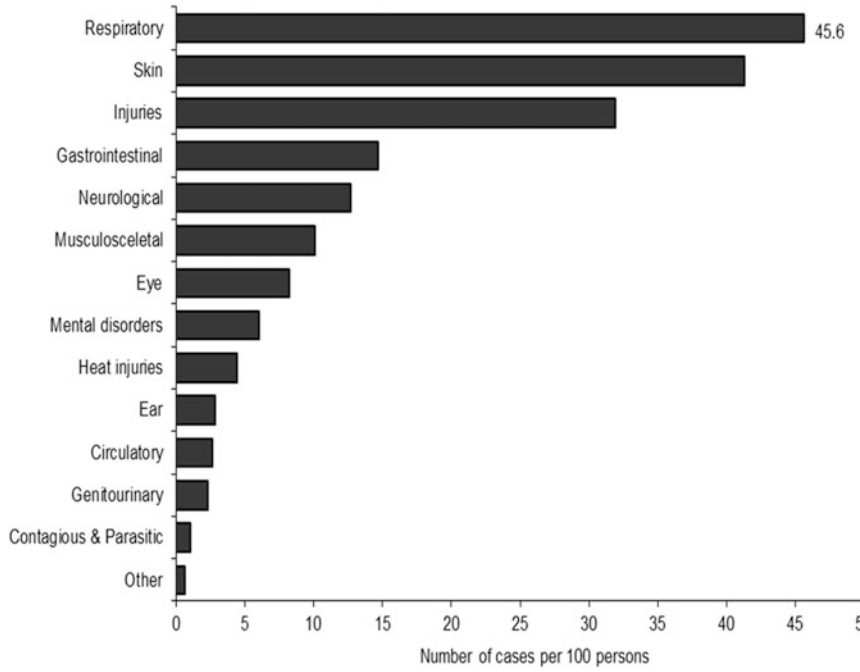

Fig. 18.1 Sickness profile in soldiers serving in the Polish Military Contingent in Iraq (n = 871), treated on an outpatient basis in Polish Field Hospital in 2003–2004

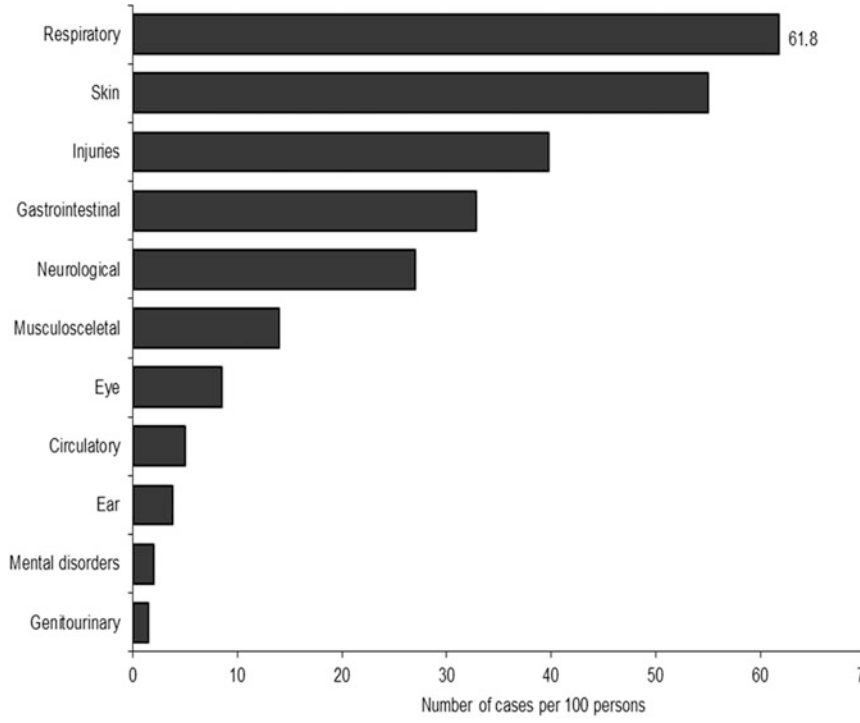

Fig. 18.2 Sickness profile in soldiers serving in the Polish Military Contingent in Afghanistan (n = 400), treated in the PMC outpatient clinic in years 2003–2005

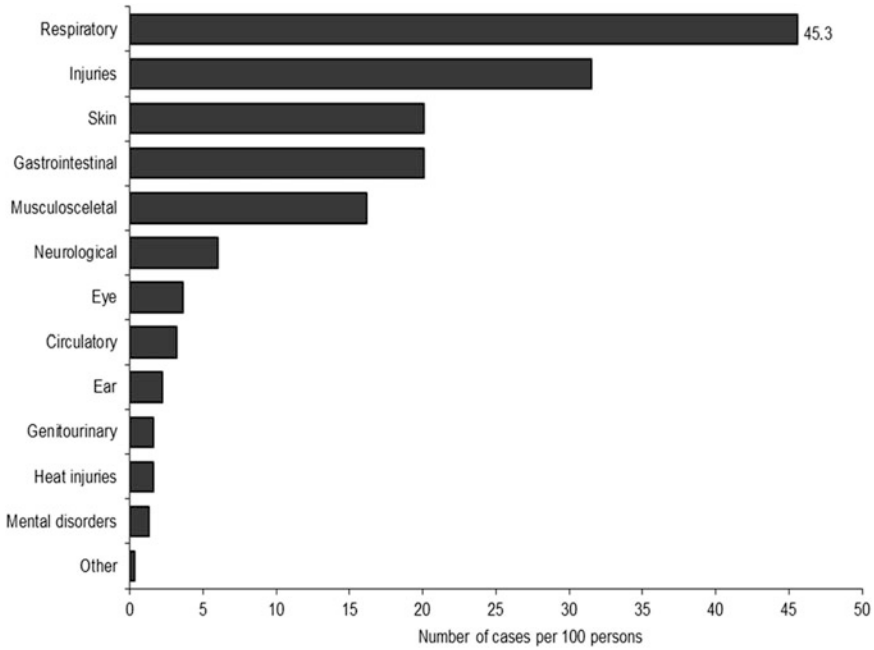

Fig. 18.3 The sickness profile in soldiers serving in the Polish Military Contingent in Afghanistan (n = 2,300), treated on an outpatient basis in Polish Field Hospital in 2009

(54.8 cases per 100 persons), injuries, skin, musculoskeletal, and gastrointestinal diseases (Fig. 18.4).

One thousand three hundred and sixty nine cases of respiratory tract illnesses were diagnosed in the population of 2,500 soldiers serving in Afghanistan in 2010. The sickness consisted of cold (33.9/100 persons), pharyngitis and/or tonsillitis (15.8/100 persons), sinusitis (3.7/100 persons), bronchitis (1.3/100 persons), pneumonia (0.1/100 persons). As always, increased prevalence of the diseases was observed in March-April and in September-October, when the Polish contingent rotated its troops.

Medical personnel supporting Polish Military Contingents in Iraq (2003–2004) and Afghanistan (2003–2005, 2009–2010) at Level 1 (outpatient medical facility) and Level 2 (field hospital) had limited diagnostic capabilities as far as the treatment of acute respiratory diseases was concerned. Bacteriological and viral diagnostic procedures were unavailable. Therefore, patients exhibiting acute respiratory symptoms routinely received antibiotics to treat the infection. As a rule, a course of antibiotics was administered only on the basis of physical examination of the patient and additional tests such as erythrocyte sedimentation rate (ESR), complete blood count (CBC), and chest and sinus X-rays.

4 Discussion

Respiratory tract diseases are one of the major health problems occurring in areas where combat operations are conducted. Such a situation is primarily influenced by difficult climatic and sanitary conditions, overpopulation, and mass migrations. High incidence of diseases is reported both in the population of immigrant soldiers participating in military operations as well as among the local people (Gray et al. 1999). *Streptococcus pneumoniae, Mycoplasma pneumoniae,* and *Haemophilus influenzae* remain the most common etiological factors causing respiratory tract diseases in both of the aforesaid populations (Gray et al. 2005; Earhart et al. 2001). During the Gulf War that took place in 1991, respiratory tract diseases were one of the most frequent health problems diagnosed among coalition forces

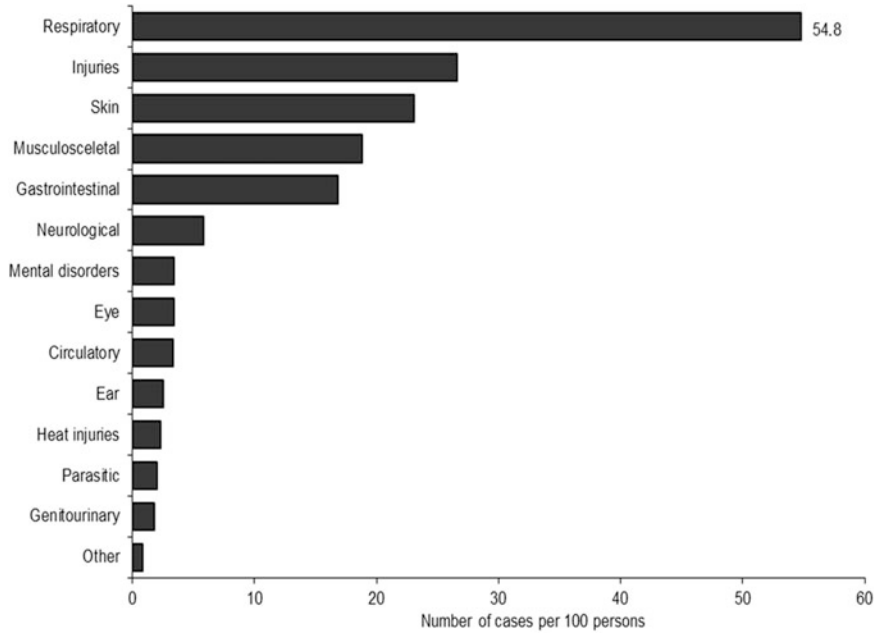

Fig. 18.4 The sickness profile in soldiers serving in the Polish Military Contingent in Afghanistan (n = 2,500), treated on an outpatient basis in Polish Field Hospital in 2010

fighting in operations *Desert Shield* and *Desert Storm* (Hyams et al. 1995). Acute respiratory tract infections, mainly in the form of bronchitis and pneumonia, represented one of the most common causes of sickness absence among Soviet troops stationing in Afghanistan in the 1980s. According to Novozhenov and Gembitski (1998), as much as 43 % of service personnel suffered from acute bronchitis and/or pneumonia within the first year of service in Afghanistan. The majority of soldiers developed respiratory tract illnesses in the fall/winter season, which was undoubtedly influenced by severe climatic conditions. Currently, military medical services put special emphasis on preventing air-borne diseases before the deployment of troops to areas of operations. Prophylactic actions are primarily based on preventive vaccinations against influenza and pneumococcal infections, and treatment by means of targeted pharmacotherapy (Crum et al. 2003; Earhart et al. 2001). The research conducted by Sanders et al. (2005) in the population of American service personnel taking part in operations *Iraqi Freedom* and *Enduring Freedom* in the period 2003–2004 also revealed that respiratory diseases remain the most frequent health problems in soldiers deployed to areas in which different climatic and sanitary conditions prevail. Sixty nine percent of the respondents complained of at least 1 episode of a respiratory tract infection and 14 % of more than 3 episodes. The incidence of respiratory tract diseases surged drastically during direct combat operations. Nearly 40 % of patients reporting respiratory diseases admitted to smoking at least 10 cigarettes per day, which, in connection with environmental conditions (extreme temperature changes within 24 h, sand, and dust storms) may notably increase the prevalence of such illnesses. Pneumonia was diagnosed in 3 % of American soldiers complaining of respiratory diseases; those patients were mainly treated on an outpatient basis (Aronson et al. 2006). Most cases of pneumonia diagnosed in the population of the U.S. Forces personnel serving in Iraq in the period March 2003–March 2004 were either of bacterial or viral etiology, and 18 patients were diagnosed with idiopathic eosinophilic pneumonia (two died) (Shorr et al. 2004).

Research conducted during the initial stage of the operation *Iraqi Freedom* in 2003 demonstrated approximately 100 cases of pneumonia among American military personnel; 15 % of which ended up in acute respiratory failure and required treatment in an intensive care unit (Gottlieb 2003; Oransky 2003). The major threat to the life and health of soldiers serving in the coalition forces relocated to Iraq and Afghanistan are undoubtedly battle injuries. Nevertheless, the prevalent health problems in the above mentioned population are different types of diseases, especially respiratory tract infections (Peoples et al. 2004). Respiratory diseases have been the major source of sickness absences, hospitalizations, and unfitness for service over the last several decades. The research conducted among American soldiers evacuated from the theater of operations for medical reasons demonstrated that more patients were evacuated due to respiratory illnesses than owing to battle injuries (James et al. 1982). Infectious diseases diagnosed in the population of military personnel assigned to contemporary armed conflicts account for merely 2.8 % of all diagnoses. This is associated with the fact that complex laboratory diagnostic procedures are unavailable inside the theater of operations. A large number of respiratory tract diseases which are diagnosed as non-infectious, may be of bacterial or viral etiology (Harman et al. 2005). Approximately 40–70 % of all military personnel staying inside the operational areas in Iraq and Afghanistan report to health care facilities due to upper respiratory tract infections. Medical personnel have no capabilities to perform bacteriological or virological diagnostic procedures. Therefore they typically administer a course of antibiotics although in some cases antibiotic treatment has no clinical justification and may facilitate the emergence of pharmacotherapy resistant microorganisms (Sanders et al. 2005).

The sickness profile observed among the Iraqi and Afghan populations is similar. Respiratory tract diseases represent the leading cause of morbidity in both adults and children less than 5 years of age (Prasad 2006; Dyer 2004). The factors which determine mass incidence of respiratory tract diseases are malnutrition, limited access to medicines and basic health care, collapse of the immunization program, migrations, and overpopulation, especially in refugee camps (Korzeniewski 2009, 2006).

If medical services operating in a mission area implemented appropriate disease prevention measures (sanitation, hygiene, and anti-epidemic support), the risk of developing of infectious or non-infectious diseases would be greatly reduced (Morris et al. 2011; Smith et al. 2009).

5 Conclusions

The prevalence of respiratory diseases among soldiers deployed to military operations in Iraq and Afghanistan was closely related to the effects of environmental factors (sand and dust storms, extreme range of temperature within 24 h, unsatisfactory sanitary conditions) and to disregard of basic principles of disease prevention.

Increased prevalence of diseases was observed throughout March-April and September-October, i.e., when the Polish contingent rotated its troops and when the newly-arriving soldiers were undergoing acclimatization process to adjust to environmental conditions prevailing in the theater of operations.

Medical personnel supporting Polish Military Contingents in Iraq and Afghanistan had limited diagnostic capabilities as far as treatment of acute respiratory diseases was concerned (bacteriological and virological diagnostic procedures were unavailable).

Acknowledgments We acknowledge the permission of the Commanders of the Polish Military Contingents in Iraq (2003–2004) and Afghanistan (2003–2005, 2009–2010) as well as the Chiefs of Military Health Services of the operations *Iraqi Freedom, Enduring Freedom* and ISAF to use medical records of military personnel for scientific publications.

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

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In Vitro Susceptibility of *Staphylococci* and *Enterococci* to Vancomycin and Teicoplanin

19

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Abstract

Hospital-acquired infections (HAIs) pose a worldwide problem. They primarily concern intensive care, hematology-oncology, and surgical units. Coagulase-positive and coagulase-negative *Staphylococci*, especially their subgroups possessing the ability to develop resistance to methicillin, and *Enterococci* have a particular role in the etiology of HAIs. The aim of this study was to determine the therapeutic minimal inhibitory concentration (MIC) values for vancomycin and teicoplanin, two of the most commonly administered antibiotics in the treatment of infections caused by *Staphylococci* resistant to methicillin, and infections caused by *Enterococci*. The material analyzed included 200 bacterial strains collected from patients treated in the Intensive Care Unit, the Musculoskeletal Infections Unit, and Surgical Clinics of the Military Institute of Medicine in Warsaw, Poland. The study was conducted in accord with the European Committee for Antimicrobial Susceptibility Testing (EUCAST) criteria by means of the Etest® gradient strips. We demonstrate a full susceptibility of *Staphylococci* MSSA (*methicillin susceptible Staphylococcus aureus*), *Staphylococci* MRSA (*methicillin*

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_19,

resistant Staphylococcus aureus), and *Enterococci* to both antibiotics. Coagulase-negative *Staphylococci* had a higher sensitivity to vancomycin. Teicoplanin had a lower MIC than vancomycin against the analyzed strains of *Enterococci*. As regards the coagulase-negative *Staphylococci*, vancomycin had a lower MIC than teicoplanin. In conclusion, the study confirmed current recommendations on the use of vancomycin and teicoplanin in the treatment of infections caused by gram-positive bacteria, emphasizing the need for the determination of MIC values.

Keywords
Enterococci • *Staphylococci* • Susceptibility • Teicoplanin • Vancomycin

1 Introduction

Hospital-acquired infections (HAIs) pose a worldwide problem. They primarily concern intensive care, hematology-oncology, and surgical units. The risk of nosocomial infections depends on the host characteristics, the number of interventions, invasive procedure, asepsis of techniques, the duration of stay in the hospital and inappropriate use of antimicrobials. Most often the endogenous flora of the patient, which may be altered because of hospitalization, is responsible for nosocomial infections (Jones 2010; Wisplinghof et al. 2004). Both coagulase-positive and coagulase-negative *Staphylococci*, especially their subgroups possessing the ability to develop resistance to methicillin, have a particular role in the etiology of HAIs (Piette and Verschragen 2009). *Staphylococci spp*. is commensal on human body surfaces and colonizes intravenous devices, which become a focus of infection in hospitalized individuals. *Staphylococci spp*. is responsible for nosocomial pneumonia, surgical site infections, bloodstream infections, and urinary tract infections (Jain and Agarwal 2009). Vancomycin remains the antibiotic of choice to treat methicillin-resistant *Staphylococcus aureus* (MRSA) infections (Pitz et al. 2011). However, due to a dramatic rise in MRSA infections and widespread use of vancomycin, which is known to have marginal tissue penetration and slow bacterial activity, MRSA strains with reduced susceptibility to vancomycin are emerging (Pitz et al. 2011). Several studies have already reported elevated minimal inhibitory concentration (MIC) for vancomycin in MRSA isolates, with MICs at the upper end of the susceptibility range (Lodise et al. 2008; Rybak et al. 2008; Hussain et al. 2002). There are increasing numbers of report indicating the emergence of vancomycin resistant *Staphylococcus aureus* (VRSA). Associated with this issue is the presence of heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA). These microorganisms are described as being susceptible to vancomycin, but contain a subpopulation that possesses a thicker cell wall and expresses resistance to vancomycin. Infections caused by hVISA are a growing concern in hospitals, resulting in prolonged bacteremia, endocarditis, and osteomyelitis and leading to vancomycin treatment failure (Cui et al. 2006, 2003).

Another group of Gram-positive pathogens that became a cause of HAIs in the 1990s are *Enterococci spp*. (Jones 2010; Wisplinghof et al. 2004). *Enterococci spp*. are intestinal commensals of humans and other animals, in addition to being isolated from environmental sources. During the past decades, enhanced prevalence of enterococcal infections emerged, such as bacteremia and urinary tract infections, along with multi-antimicrobial resistance, particularly vancomycin resistant enterococci (VRE) (Courvalin 2006; Cetinkaya et al. 2000). Among this group of bacteria, especially *Enterococcus faecium* has a mechanism for developing

resistance to vancomycin. Six different glycopeptide-resistant phenotypes (*VanA* to *VanE* and *VanG*) have been described in enterococci, while *VanA* and *VanB* are of greatest clinical relevance. Strains resistant to vancomycin and teicoplanin have been assigned to Van A phenotype, while those susceptible to teicoplanin but resistant to vancomycin are considered as the *VanB* phenotype (Thierfelder et al. 2012; Courvalin 2006; Cetinkaya et al. 2000).

Therapeutic success in the treatment of infections depends not only on determining the susceptibility of given bacteria to antibiotics (determined on the basis of the upper cut-off level for MIC), but also on identifying absolute MIC value in a wide range of concentrations (Hryniewicz 2000). The aim of the present study was to determine the therapeutic MIC values for vancomycin and teicoplanin, two of the most commonly administered antibiotics in the treatment of infections caused by *Staphylococci spp.* and infections caused by *Enterococci*.

2 Methods

The study was approved by the Ethics Committee of the Military Institute of Medicine in Warsaw, Poland. The biological material analyzed was collected from patients treated in the Clinical Intensive Care Unit, the Musculoskeletal Infections Unit, and Surgical Clinics of the Military Institute of Medicine in Warsaw in the period April-July 2011. The source of the samples analyzed included cultures from blood (14 samples), bronchial tree (14 samples), peritoneal cavity (7 samples), wounds (123 samples), abscesses (20 samples), and ulcerations (22 samples). Two hundred strains of Gram-positive bacteria were subject to analysis, including 50 *Enterococci* strains (39 strains of *Enterococcus faecalis* cultured from blood – 1, peritoneal cavity – 4, wounds – 29, abscess – 1, ulcerations – 4; 11 strains of *E. faecium* cultured from peritoneal cavity – 3, wounds – 7, abscess – 1); 89 strains of *Staphylococcal aureus* MSSA (*methicillin-susceptible S. aureus*, cultured from blood – 7, bronchial tree – 8, wounds – 47, abscess – 13, ulcerations – 14); 24 strains of *S. aureus* MRSA (*methicillin-resistant S. aureus,* cultured from blood – 3, bronchial tree – 6, wounds – 12, abscess – 1, ulcerations – 2); and 37 strains of coagulase-negative *Staphylococci,* (cultured from blood – 3, wounds – 28, abscess – 4, ulcerations – 2; including 14 strains of MSCNS, *methicillin-susceptible coagulase negative Staphylococcus* and 23 strains of MRCNS, *methicillin-resistant coagulase negative Staphylococcus*).

The species of isolated bacterial strains were identified using the VITEK 2 system (bioMérieux, France) by means of GN cards, following the guidelines issued by the manufacturer. The strains which were identified were next subject to manual determination of MIC value of glycopeptides by means of Etest® gradient strips which measure the concentration of a given antibiotic on the Müeller-Hinton plates (bioMérieux, France). The range of concentrations analyzed, for both vancomycin and teicoplanin, was from 0.032 to 256.0 μg/mL. The analysis was carried out in compliance with recommendations from the National Reference Center for Drug Susceptibility of Microorganisms in Poland. The results of the research were interpreted in line with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria, which have been legally binding in Poland since 1 April 2011. Reference strains *Staphylococcus aureus* ATCC29213 and *Enterococcus faecalis* ATCC 29212 were used to control test strips. MIC limiting values used to classify a strain as either susceptible or resistant according to EUCAST guidelines are presented in Table 19.1. A commercial packet of Statsoft Statistica 9 was used to perform all calculations.

3 Results

Data regarding the exact number and type of particular bacterial strains, and their susceptibility or resistance to the glycopeptides studied are presented in Table 19.2. Table 19.3 illustrates the distribution of vancomycin and teicoplanin MIC values against different bacterial strains.

Table 19.1 Interpretation of MIC limiting values for Gram-positive cocci in accord with EUCAST criteria

	Vancomycin μg/mL		Teicoplanin μg/mL	
Bacterial strain	S≤	R>	S≤	R>
Enterococcus (*E. faecium*, *E. faecalis*)	4	4	2	2
S. aureus	2	2	2	2
S. aureus MRSA	2	2	2	2
Coagulase-negative *Staphylococci*	2	2	4	4

S susceptible, *R* resistant

Table 19.2 The number of isolates obtained and their susceptibility to glycopeptides

		Vancomycin		Teicoplanin	
Bacterial strain	Number of isolates	S	R (%)	S	R (%)
E. faecium	11	11	0	11	0 (0)
E. faecalis	39	39	0	39	0 (0)
S. aureus MSSA	89	89	0	89	0 (0)
S. aureus MRSA	24	24	0	24	0 (0)
Coagulase-negative *Staphylococci*	37	37	0	31	6 (16.2)
Total	200	200	0	194	6 (3.1)

S susceptible, *R* resistant

Data presented in Table 19.2 confirm high efficacy of both vancomycin and teicoplanin against *Enterococci spp*. and *Staphylococci spp*. However, as regards coagulase-negative *Staphylococci*, teicoplanin has not been effective towards 16.2 % of the bacterial strains analyzed.

According to the values presented in Table 19.4, teicoplanin exhibits lower MIC 50 and MIC 90 values in case of *Enterococci*. No such difference was noted as regards MRSA. MIC 50 of teicoplanin in regard to *S. aureus* MSSA was slightly lower. Large differences were observed for *E. faecium*, for which MIC 50 and MIC 90 values of teicoplanin were lower and thus more favorable. With regard to the coagulase-negative *Staphylococci*, vancomycin exhibited much lower MIC 50 and MIC 90 values than teicoplanin.

4 Discussion

Our data indicate good susceptibility of *Enterococci spp*. and *Staphylococci spp*. to vancomycin, as none of isolated strains were resistant to it, which is consistent with the results obtained in other studies (Nimmo et al. 2011). *Enterococci spp*., MSSA and MRSA, were also fully susceptible to teicoplanin, while 16.2 % of coagulase-negative *Staphylococci* were resistant to it. The proportion of the coagulase-negative staphylococci resistant to teicoplanin in our study seems high when compared with that observed in other studies. Ma et al. (2011) found the increasing trend in the prevalence of strains of coagulase-negative *Staphylococci* non-susceptible to teicoplanin (from 4.5 to 6.7 %).

Our present results relating to coagulase-negative *Staphylococci* need to be discussed separately. The bacteria demonstrate exceptional ability to adhere to synthetic materials. Therefore, these materials are the main source of infection spread through central venous catheters and the like, inserted into the patient's body. Such cases were reported in the Musculoskeletal Infections Unit, in which the cultures to be analyzed were collected from patients with artificial joints or those with fractures that had been stabilized using bonding materials. Out of the 37 strains of coagulase-negative *Staphylococci*, 23 developed resistance to methicillin. As mentioned above, 16.2 % of all studied coagulase-negative

Table 19.3 Distribution of vancomycin and teicoplanin MIC values in different bacterial strains

		The number of strains, against which MIC (μg/mL) amounted to:																
Bacterial strain	Antibiotic	0.032	0.47	0.94	0.125	0.19	0.25	0.38	0.50	0.75	1.0	1.5	2.0	3.0	4.0	6.0	8.0	256.0
Enterococci	Vancomycin	0	0	0	0	0	0	4	3	2	15	16	5	5	0	0	0	0
	Teicoplanin	0	1	9	20	12	3	1	2	2	0	0	0	0	0	0	0	0
E. faecium	Vancomycin	0	0	0	0	0	0	2	2	1	6	0	0	0	0	0	0	0
	Teicoplanin	0	0	3	1	3	1	1	2	0	0	0	0	0	0	0	0	0
S. aureus MSSA	Vancomycin	0	0	0	0	0	0	0	4	11	54	12	8	0	0	0	0	0
	Teicoplanin	0	0	0	0	3	6	15	27	9	15	7	7	0	0	0	0	0
S. aureus MRSA	Vancomycin	0	0	0	0	0	0	2	5	6	8	3	0	0	0	0	0	0
	Teicoplanin	0	0	0	0	0	0	1	3	9	8	3	0	0	0	0	0	0
Coagulase-negative *Staphylococci*	Vancomycin	0	0	0	0	0	0	0	0	5	14	16	2	0	0	0	0	0
	Teicoplanin	0	0	7	0	0	3	1	2	0	1	6	10	1	3	2	1	0

Table 19.4 Comparison of MIC 50 and MIC 90 values and MIC ranges for the bacterial strains studied

Bacterial strain	Antibiotic	MIC 50	MIC 90	MIC ranges
Enterococci	Vancomycin	1.5	2.0	0.38–3.0
	Teicoplanin	0,125	0.25	0.047–0.75
E. faecium	Vancomycin	1.0	1.0	0.38–1.0
	Teicoplanin	0.19	0.5	0.094–0.5
S. aureus MSSA	Vancomycin	1.0	1.5	0.50–2.0
	Teicoplanin	0.5	1.5	0.19–2.0
S. aureus MRSA	Vancomycin	0.75	1.5	0.38–1.5
	Teicoplanin	0.75	1.5	0.38–1.5
Coagulase-negative Staphylococci	Vancomycin	1.0	1.5	0.75–2.0
	Teicoplanin	1.5	4.0	0.094–8.0

Staphylococci exhibited resistance to teicoplanin, which is a common phenomenon as regards this type of infections (Piette and Verschragen 2009). Our data presented in Table 19.4, where MIC 50 and MIC 90 values are lower for vancomycin than for teicoplanin, confirm the above statement. Data regarding susceptibility of MSSA to vancomycin and teicoplanin are of little clinical significance as administration of semi-synthetic penicillin or first-generation cephalosporins is a much better therapeutic choice (Piette and Verschragen 2009). According to our results, vancomycin is still a good antibiotic choice for patients suspected of infections with *Enterococci spp*. and *Staphylococci spp*.

In general, there is a wider range of possibilities for treating Gram-positive than Gram-negative bacterial infections. Currently, apart from the classic antibiotics, discussed in the present article, used to treat Gram-positive infections, new substances such as linezolid or tigecycline have been more commonly used. In addition, a number of new betalactam antibiotics, exhibiting high activity against MRSA, are under phase 2 and phase 3 clinical trials (Hryniewicz 2000). However, according to one of the published analyses, the effects of treating MRSA pneumonia with linezolid were no better than those produced by administration of vancomycin or teicoplanin (Kalil et al. 2010).

MRSA exhibiting reduced vancomycin susceptibility have recently been reported in many countries (Cui et al. 2003, 2006). In such a case, an increase in the dose of the antibiotic, even up to 4 g/24 h, may be required to achieve vancomycin concentration in the serum of 20–30 μg/mL (Smuszkiewicz et al. 2007). This situation was not observed while conducting the present study. The majority of the MRSA strains obtained from the patients proved to be susceptible to vancomycin and teicoplanin at equal values of MIC 50 and MIC 90. In response to increasing presence of vancomycin MICs and MRSA isolates, the Infectious Diseases Society of America, the American of Society of Health-Systems Pharmacists, and the Society of Infectious Diseases Pharmacists developed weight-based dosing recommendations for vancomycin based on pharmacokinetic and pharmacodynamic data (15–20 mg/kg i.v., every 12 h), but a recent multicenter study has not confirmed a reduced mortality rate associated with the empiric use of weight-based, guideline-recommended vancomycin dosing (Hall et al. 2012).

It has been previously discussed that nosocomial enterococcal infections pose a considerable health problem, especially if the source of infection is located in the lower part of the digestive tract. *Enterococci* accounted for 25 % of the isolates discussed in this article (including 39 strains of *E. faecalis* and 11 strains of *E. faecium*). According to the values presented in Table 19.2, teicoplanin is a better therapeutic choice in the treatment of enterococcal infections than vancomycin. The distribution of MIC values indicates that, regarding *Enterococci* (including *E. faecium*), the teicoplanin MIC 50 and MIC 90 values are much lower, and hence more favorable, compared with vancomycin. Teicoplanin is preferred in the treatment of enterococcal infections as it reduces the risk of a therapeutic

failure, which may arise due to the emergence of *VanB*- and *VanE*-genotype resistance. Administration of vancomycin in such cases could facilitate the development of vancomycin-resistant of *Enterococci*. Teicoplanin penetrates into tissue more easily than vancomycin whose serum concentration in lungs amounts to 30 %. While studying the levels of teicoplanin in pulmonary alveoli mucus, it has been determined that the optimum therapeutic dose of intravenous teicoplanin is 12 mg/kg twice on the first day, followed by 12 mg/kg daily later on (Mimoz et al. 2006). In patients suffering from severe sepsis or a septic shock, who are given large volume of infusion fluids or receive catecholamines, penetration of a drug is reduced due to vessel shrinkage. As a result, the concentration of a drug in tissue is below the therapeutic level, although MIC values in the serum are satisfactory (Joukhadar 2001).

The present study confirmed current recommendations on the use of vancomycin and teicoplanin in the treatment of infections caused by Gram-positive bacteria, emphasizing the need for the determination of MIC values.

5 Conclusions

On the basis of MIC 50 and MIC 90 values the following conclusions can be drawn: (1) teicoplanin exhibits higher clinical efficacy in the treatment of infections caused by *Enterococci*; (2) efficacy of vancomycin and teicoplanin to *S. aureus* MSSA and MRSA is comparable; (3) vancomycin exhibits more favorable MIC values than teicoplanin in regard to the coagulase-negative *Staphylococci*.

Acknowledgments We thank Dr. Dariusz Tomaszewski from the Clinic of Anesthesiology and Intensive Care of the Military Institute of Medicine in Warsaw, for stylistic editing of the manuscript.

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

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Sinusitis in People Living in the Medieval Ages

20

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Abstract

Breathing vitally serves body homeostasis. The prevalence of upper airway infections is often taken as an indicator of overall health status of a population living at a given time. In the present study we examined the unearthed remains of skulls from the XIII-XV century inhabitants searching for signs of maxillary sinusitis. Maxillary sinuses of the skulls of 92 individuals were inspected macroscopically and, if necessary, endoscopically. Osseous changes, including the pitting and abnormal spicule formation were present in 69 cases (75.0 %). It was found that, overall, dental infection was a major cause of maxillary sinusitis (18.8 %). Severe bone changes were observed in the adults' skulls, but were also present in the sinus walls of children's skulls. Post-inflammatory changes were manifest as remodeling and damage to the sinus walls. The results indicate that both children and adults of the Middle Ages suffered from chronic sinusitis. These observations confirm that the climate, environment, and lifestyle of the medieval populations contributed to the morbidity of the upper respiratory tract.

Keywords

Allergization • Endoscopy • Maxillary sinus • Medieval population • Mucositis • Sinusitis • Skull

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_20,

1 Introduction

Breathing depends on clear sinuses, and the occurrence of sinusitis is particularly unpleasant. The paranasal sinusitis is a disease that afflicts a significant percentage of population and causes considerable long-term morbidity. A large part of the contemporary urban and rural populations suffer from mucositis of the nose and paranasal sinuses. It is estimated that this applies to about 12 % of population. Undoubtedly, environmental pollution and increasingly common allergization by natural and chemical environmental factors increase the occurrence of sinusitis. Both indoor and outdoor conditions were understandably different in the middle ages, with likely less outside environmental but more indoor pollution; the latter stemming from wood-burning, a source of emission of oxides of carbon and nitrogen, fine particulates, volatile organic compounds, and sulphur dioxide, combined with improper ventilation and biocontamination due often to living together with domestic animals (Engelhart et al. 1999). Contemporary studies demonstrate about 20 % incidence of chronic bronchitis which may have to do with poorly ventilated houses. The upper airway complaints particularly concern women due to their role in housekeeping and cooking (Roberts 2007; Shrestha and Shrestha 2005; Brauer et al. 1996). Respiratory diseases and their consequences were surely the cause of sickness in medieval human populations as well. The prevalence of such diseases is often taken as an indicator of health status (Benninger 2003), but their incidence in medieval times is largely unknown. In the present study, therefore, we set out to examine the skulls of people living in medieval times searching for signs of chronic inflammation in the maxillary sinus.

2 Methods

The study was approved by a local Ethics Committee. A total of 105 skulls unearthed from two XIII-XV century cemeteries belonging to the Order of the Knights Templar in the villages of Rurka and Chwarszczany (northwestern region of Poland) were examined in this study. The Order's manor house in the village of Rurka was the center of the Knights' commandery. The village chapel and a nearby cemetery served both the knights and their servants, which is reflected in the consecutively superimposed strata of the Order's and secular burial layers at the cemetery. The Rurka commandery population was represented by 90 skulls (169 maxillary sinuses). The Rurka Order's headquarters were situated in the flat, ellipsoidal area of a Rurzyca river bend, surrounded by marshes and wetlands and were situated in an area otherwise inconvenient for the purpose of a common settlement. The Order's housing and offices were slightly better than those belonging to the commonalty, but they were largely typical for the time, with small or no windows – reducing the supply of ventilation and light and with household flock living together. A comparative sample from Chwarszczany consisted of 15 skulls (30 maxillary sinuses) of children, females, and males, and belonged to individuals aged 2–80. The inhabitants of this village apparently belonged to the Knights' attendants and operating personnel and, judging from a very modest and simple burial outfit, belonged to a lower socio-economic class.

The assessment of sex and age was done according to criteria worked out by Ubelaker (1989). Briefly, gender was determined from the assessment of cranial morphology such as: the prominence of glabella, sharpness and thickness of the supra-orbital margins, the robustness of the nuchal crest, and the size of the mastoid processes. The age at death was estimated using the following criteria: dental eruption and occlusion, cranial suture closures, postcranial epiphyseal unions, and pubic symphyseal face morphology. The appearance of the anterior and posterior fontanelles and fusion of the suture lines was used to estimate age in the perinatal period and the general sequence of eruption of the deciduous and permanent teeth in older infants and children.

The maxillae were sorted by age and, whenever possible by gender of an individual. We studied the maxillary sinus macroscopically and

with a rigid endoscope. In complete skulls, the endoscope was introduced in the sinus through the nasal cavity. Changes in the sinus walls caused by the inflammatory process were classified according to the 'morphological type': isolated spicules and clusters of interconnected spicules, pits, cysts, lobules, and the surface structure and thickness of the bony reaction (Roberts 2007; Merrett and Pfeiffer 2000; Ortner 2003). Eleven intact skulls were trephined through the posterior wall of the maxillary sinus to obtain access to the interior of sinuses. A 4 mm diameter, rigid endoscope was used to visualize the maxillary sinus lumen. The signal was recorded on computer and archived for off-line analysis. We investigated dental pathology such as: periapical abscesses, oroantral fistulae, evident periodontitis, and *ante mortem* tooth loss. Statistical analyses were done by using Microsoft Excel, version 2010. A $p < 0.05$ was considered significant for all comparisons.

3 Results

Ninety two skulls out of the 105 unearthed were suitable for further analysis. There were 13 female and 56 male skulls belonging to adult individuals; gender could not be conclusively defined in the children group (Table 20.1). In 69/92 (75 %) cases, the maxillae showed changes in bone morphology consistent with sinusitis. Overall, inflammation-related odontogenic changes were seen in 13/69 (18.8 %) of cases. Therefore, lack of inflammatory changes or ambiguous unsettled changes were found only in 25 % of cases. In the village of Rurka there was a high percentage of skulls belonging to children and young individuals with the signs of sinusitis (Figs. 20.1 and 20.2). Otherwise, however, there were little variations between the results obtained from the two locations.

The correlation between age and the occurrence of sinusitis was significant (χ^2 9.75, df. 2; $p = 0.005$). There also was a correlation between increasing age and dental pathology (χ^2 11.33, df. 2; $p < 0.001$). In the infant and children's maxillary sinuses examined, there were relatively abundant scarring marks seen in 17\23 (73.9 %); these marks were particularly evident in the skulls belonging to infants. In the sinus walls, there were a great deal of spicules, their combinations, or some form of remodeling of the wall as a result of the inflammatory process. These changes and their types were present in different proportions (Fig. 20.1). Spicules, lobules, the surface and thickness changes represent the bony reaction in the form of bone deposition, while pits and cysts reflect bone resorption (Figs. 20.3 and 20.4). The skulls of males from the village of Chwarszczany had significantly less signs of passed sinusitis. There were no statistical differences in the prevalence of remodeling and abscessing, or remodeling independent of abscessing, between the female and male samples examined, although the abscessing and dental pathologies tended to more frequently observed in male skulls (Fig. 20.2). Other differences were either less appreciable or obscured by demographic discrepancies.

Table 20.1 Morphological changes in maxillary sinuses

Age category (year)	Female	Male	Remodeling n(%)	Dental pathology n(%)
Infants (0–6)	16		12 (11.4)	0
Children (7–15)	7		5 (4.8)	0
Adolescents (16–22)	0	5	2 (1.9)	2
Young adults (23–40)	10	12	16 (15.3)	2
Middle-aged adults (41–59)	2	32	28 (26.7)	4
Seniors (60+)	1	7	5 (4.8)	5
Total	13	56	69 (75)	13 (18.8)
	92			

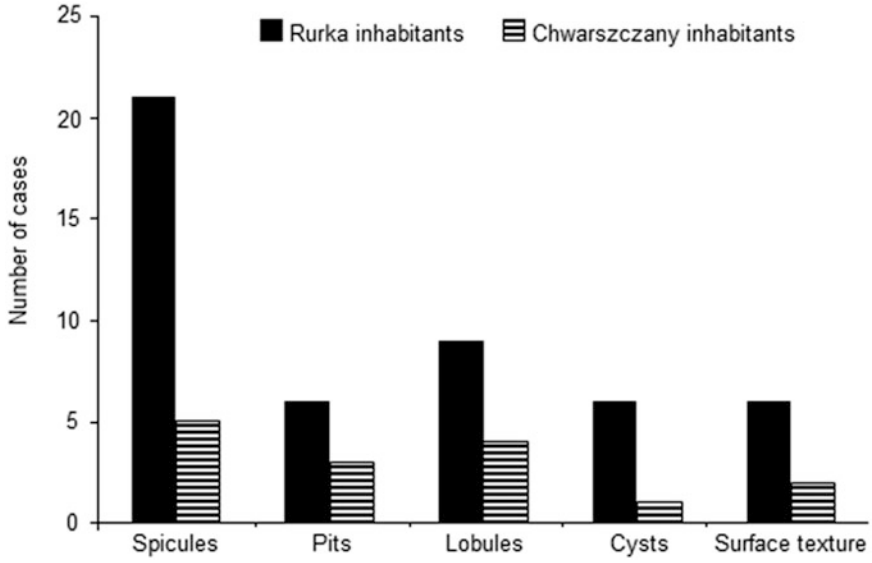

Fig. 20.1 Proportion of osseous lesions observed in maxillary sinuses in the XIII-XV century skulls unearthed in the villages of Rurka and Chwarszczany

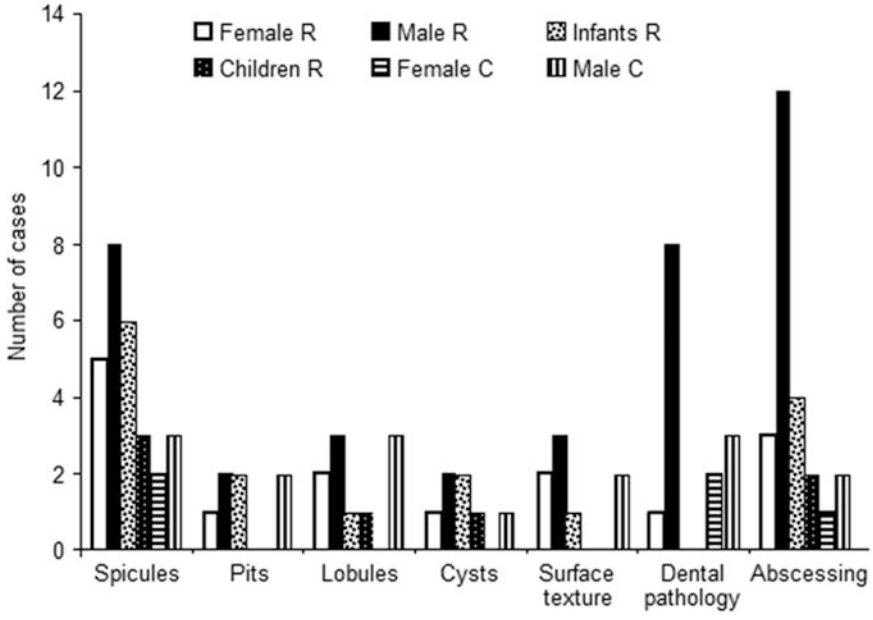

Fig. 20.2 Demographics concerning the XIII-XV century skulls unearthed in the villages of Rurka (*R*) and Chwarszczany (*C*), with the signs of remodeling, abscessing, and various osseous lesions

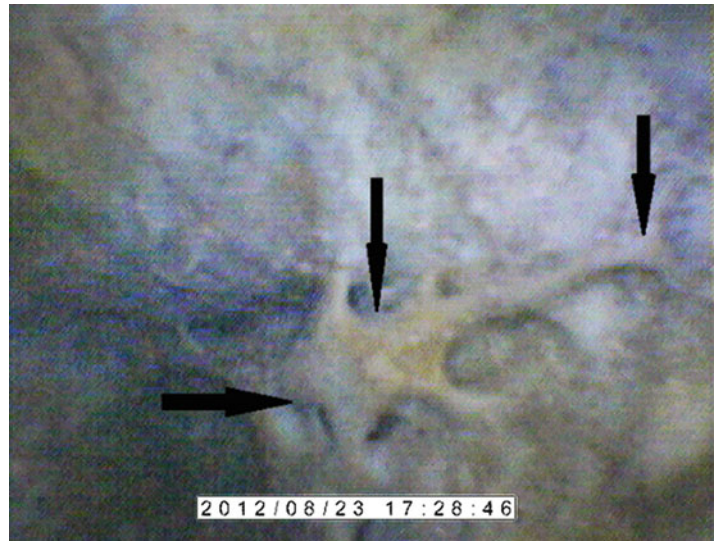

Fig. 20.3 Clusters of interconnected spicules in a corner of the upper posterior wall of an adult left maxillary sinus (endoscope captured image)

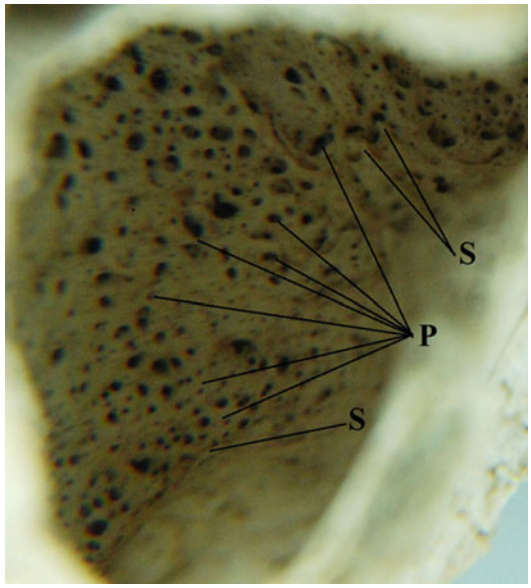

Fig. 20.4 Left maxillary sinus (adult male from the Rurka village, with sings of dental pathology); *S*-spicules and *P*-pits of the bony reaction in the maxillary floor

4 Discussion

The finding of the present study was that a substantial proportion of medieval inhabitants of villages in a northwestern region of Poland, despite being of a higher-than-average socio-economic status, suffered from maxillary sinusitis. Permanent traces of sinusitis in the form of sinus bone and dental remodeling were found in three thirds of the skulls examined. The corollary is that upper airway complaints must have been common in those times and the resistance of the body against airway infections was no higher than it is contemporarily.

Studying health status across ages is a unique way of understanding of how people adapted to changing socio-economic and political environments through the times, what their health condition was, and how that affected the ability to function in their communities (Merrett and Pfeiffer 2000). Upper airway diseases, most notably infections are often taken as a surrogate of respiratory health in both past and contemporary populations (Benninger 2003). The upper airways are special in this assessment of past generations' health in that airways are the first line of defense against the environmental offenders and as such underlie body homeostasis. An inflammatory process often initiates in, and spreads from, the nasal cavities. The narrow sinus ostia are passages that easily get obstructed due to swelling and mucosal adenomatous hyperplasia developing around the ostiomeatal complex (Ramanathan and Lane 2007). These factors induce inflammation of the

paranasal sinuses and mucous membrane lining them, but also affect the bonny walls in the course of time. The spongy skull structure contributes to the inflammatory complications (Perloff et al. 2000). Bones are subject to inflammatory remodeling, which also leads to sustained swelling of the adhering mucosa (Khalid et al. 2002). Thus, osteitis is a factor in the etiology of sinusitis. Some authors believe that the thickening of sinus walls and bone sclerosis underlie a chronic, years-long process of sinusitis (Perloff et al. 2000). The present study on the maxillary sinuses representing a sample of inhabitants of the medieval times confirms the theory that inflammatory changes in the mucosa of paranasal sinuses are reflected in bone inflammation.

Other studies on the status of maxillary sinuses in medieval populations suggest a possible relation between then-present living conditions, and air and climate quality and the incidence of sinusitis (Lewis et al. 1995; Roberts 2007). Those studies point to such potential factors facilitating sinusitis as pollution from the use of biomass fuels for cooking and heating, concentration of indoor pollutants caused by living together with domestic animals, or high humidity while living close to water resources. All those factors also were likely present in the environment of the inhabitants of the two villages whose skulls were examined in the present study for the remnant signs of passed sinusitis. A higher incidence of sinusitis in men could correspond to their being more exposed to the outside climatic conditions and weather changes. Also, a large number of changes in the sinuses of children suggest their low resistance to chronic pathologies of upper airways.

A small size of the sample, complex etiology of sinusitis, and difficulties in determining the presence of sinusitis make one interpret the results with caution. Despite all the inherent limitations of trying to get insight into the health status of the inhabitants of the time long bygone, we believe we may conclude that chronic upper airway pathologies were a frequent accompaniment in the medieval times as they continue to be so. Analysis of the whole sample also demonstrates that the prevalence of sinusitis increased with advancing age of the people of the time.

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

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Immunization Coverage Against Capsular Bacteria in Splenectomized Patients

21

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Abstract

Splenectomy significantly increases the risk of severe invasive infections caused by capsular bacteria, such as sepsis and meningitis. Immunizations before and after splenectomy reduce the risk and are routinely recommended. Little is known about compliance with actual immunization guidelines in Poland. The aim of this study was to analyze the vaccination rate and the knowledge of splenectomized patients concerning immunizations in Poland. We applied a questionnaire to survey 85 adult patients (F/M 49/36) splenectomized in 2009–2010 and analyzed the patients' medical files and immunization certificates. Patients were also questioned over the phone. We found that the patients were most commonly immunized against *Streptococcus pneumoniae* (17/85, 20 %), less often against *Haemophilus influenzae* b (8/85, 9.4 %), and rarely against *Niesseria meningitidis C* (3/85, 3.5 %). In contrast, hepatitis B immunization coverage rate was as high as 67 % (57/85). The majority of respondents (59/85, 69.4 %) regarded information about the recommended immunizations as insufficient and rated their doctor's reasoning as inconsistent, a smaller number (20/85, 23.5 %) confirmed they received sound information before splenectomy. Both surgeons and primary care physicians did not offer immunizations to the majority of patients (59/85, 69.4 %); as a result, only 30.6 % of patients (26/85) were

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_21,

immunized against any capsular bacteria before splenectomy. In conclusion, the majority of splenectomized patients are not immunized despite current guidelines and do show an inadequate level of knowledge concerning the consequences of splenectomy. It is important that both surgeons and primary care doctors give patients clear instructions about immunizations and antibiotics recommended before and after their splenectomy.

Keywords
Asplenia • Immunization • Infection prophylaxis • Risk factor

1 Introduction

Splenoctomized patients, as a result of the immunity impairment, are at high risk of developing infections with capsular bacteria (0.23–0.42 % annually, with a cumulative lifetime risk of about 5 %) with the mortality rate as high as 50 %; so-called overwhelming post-splenectomy infection (OPSI) (Di Sabatino et al. 2011; Stanford et al. 2009; Shatz 2005; Davidson and Wall 2001). The particularly high risk is connected to the infection with *Streptococcus pneumoniae*, *Haemophilus influenzae* type b and *Meningococcus* of serogroup C (Di Sabatino et al. 2011; Fuentes-Ferrer et al. 2008). *Streptococcus pneumoniae* alone is responsible for more than 50 % of cases (Bird 2010; Aguilar et al. 2010; Jones et al. 2010; El-Alfy and El-Sayed 2004; Davidson and Wall 2001). The risk can be reduced by immunizing patients and preventive administration of antibiotics (Di Sabatino et al. 2011; Oller-Sales et al. 2011; Lammers et al. 2010; Zarina et al. 2010). Vaccinations against *Streptococcus pneumoniae, N. meningitidis* group C, *Haemophilus influenzae* type b, and influenza in splenectomized patients are universally recommended in the European Union countries. Preventive antibiotic therapy for 2–5 years in children and 2 years in adults is also suggested (Di Sabatino et al. 2011; Coignard-Biehler et al. 2008). However, the comparison of the European and US guidelines clearly shows a lack of conclusive, unambiguous recommendations. Despite the existing recommendations, the decision concerning immunization and subsequent preventive antibiotic therapy is undertaken more often in patients who undergo a pre-scheduled splenectomy due to hematologic disorders than in acute traumatic patients (Di Sabatino et al. 2011; Lamsfus-Prieto et al. 2007).

In Poland, current guidelines recommend the assessment if all obligatory immunizations are up to-date and additional vaccinations against *Streptococcus pneumoniae, N. meningitidis* group C, *Haemophilus influenzae* type b, hepatitis B, and influenza. After splenectomy, a booster vaccination against the above-mentioned bacterial strains is recommended. Prophylaxis with oral penicillin or erythromycin should be considered depending on the reason for the splenectomy. Patients should be informed about the increased risk of life-threatening infections, and be given an identity card and medical certificate with information about asplenia and recommendations in case of infection. Immunizations should be given at least 2 weeks before the scheduled splenectomy. In situations where this is not possible, vaccinations should be given 2 weeks after splenectomy. Booster doses are recommended every 5 years. These recommendations are consistent with the guidelines of other EU countries (Spoulou et al. 2011; Coignard-Biehler et al. 2008; Salisbury et al. 2006). Vaccinations for splenectomized patients are however neither obligatory nor reimbursed by public funds. This could be one of the reasons that they are often neglected.

The aim of this study was to analyze the vaccination rate, the knowledge of splenectomized

patients concerning immunizations in Poland, and to determine compliance with recommendations and identify existing barriers to immunizations.

2 Methods

The study was approved by an institutional Review Board for Human Research. We assessed 85 adult patients (F/M 49/36, mean age 45.5 years) from the Department of General and Hematological Surgery of the Institute of the Hematology and Transfusiology in Warsaw, Poland, who were splenectomized in 2009–2010. Thirty six patients were hospitalized in 2009 (including 21 women) and 49 patients (including 28 women) in 2010. There were 7 (8.2 %) patients aged 18–20 years, 18 patients aged 21–30 (21.1 %), 11 patients aged 31–40 (12.9 %), 10 patients aged 41–50 (11.8 %), 16 aged 51–60 (18.8 %), and 24 aged 61 and over (28.2 %).

All patients were assessed using a standard questionnaire and their medical files and charts, including vaccination certificates were analyzed. Patients were also questioned over the phone. The reasons for the splenectomy were: idiopathic thrombocytopenia (29/85, 34.1 %), lymphoma (19/85, 22.4 %), spleen cysts (14/85, 16.5 %), spleen tumors (4/85, 5.9 %), congenital haemolytic anemia (4/85, 5.9 %), spleen infarct, chronic lymphocytic leukaemia (2/85, 2.4 %), and other single cases, e.g., spleen abscess, aneurism of spleen artery, vascular abnormality, subcapsular hematoma, exudative changes to the spleen, enlarged spleen, spleen rupture as a complication of the infectious mononucleosis (11/85, 12.9 %). Significantly more patients came from small cities and villages (50, 58.8 %) than from large cities (35, 41.1 %; $p < 0.05$). In most cases, splenectomy was performed as a scheduled procedure (61/85, 71.8 %) rather than in an emergency (defined as surgery within 2 months of first presentation in the surgical department: 24/85, 28.2 %; $p < 0.05$). Splenectomy was preferably performed by a laparoscopic method, in this case, patients were hospitalised for 10 days, or due rarely to a large spleen or underlying disease by classical surgery, with hospitalization for 10–14 days, providing there were no complications. Although preoperative antibiotics were routinely administered, two patients developed severe infections with capsular pathogens and were treated for 30–40 days before passing away in the Intensive Care Unit.

Categorical variables were presented as absolute (n) and relative (%) frequencies. Differences were regarded as statistically significant if $p < 0.05$. A commercial Statistica ver. 9.0 package was used for all data elaboration.

3 Results

3.1 Vaccination Coverage

Only were 30.6 % of the patients (26/85, 14 from villages and small cities and 12 from large towns) vaccinated against capsular bacteria before splenectomy. The patients were most commonly immunized against *Streptococcus pneumoniae* (17/85, 20.0 %), less often against *H. influenzae* type b (8/85, 9.4 %), and rarely against *N. meningitidis* C (3/85, 3.5 %). Hepatitis B vaccination coverage rate was as high as 67.1 % (57/85).

3.2 Patients' Knowledge and Barriers to Immunizations

The majority of patients 59/85 (69.4 %) could not name any capsular bacteria and 46/85 (54.1 %) knew nothing about immunizations and infectious dangers related to asplenia. The remaining patients, 39/85, including all 26 immunized patients declared that they were aware of the importance of immunizations before their splenectomy, but their knowledge was highly unspecific; they usually indicated hepatitis B as the most significant threat related to splenectomy. Out of the 39 patients informed about vaccinations, 26 (66.7 %) were immunized and another 5 (12.8 %) were probably immunized (documented data were missing), 5 (12.8 %)

Table 21.1 Intentions of patients concerning immunizations after splenectomy; results of a phone-based inquiry of 85 patients

	Streptococcus pneumoniae	*Neisseria meningitidis group C*	*Haemophilus influenzae type b*
Results of inquiry	n (%)	n (%)	n (%)
Patients did not yet make up his mind about vaccination	20 (71.4)	20 (80.0)	30 (93.7)
Patients were immunized	8 (28.6)	5 (20.0)	2 (6.3)

were not immunized due to a high vaccine cost, and 3 (7.7 %) were not immunized due to lack of confidence and misconceptions about vaccinations. The majority of patients (59/85, 69.4 %) were not informed about vaccinations against capsular bacteria by their doctors and knew nothing either about preventive antibiotics or about the urgency of contacting their primary care physician in case of fever. When questioned about the availability of information about immunizations, these 59/85 patients regarded information as insufficient and rated their doctor's reasoning as inconsistent. Only did 20/85 (23.5 %) respondents confirm good availability of information from their doctors. Both surgeons and primary care physicians did not offer immunizations recommended before a scheduled splenectomy; as a result only double 30.6 % of patients (26/85) were immunized against capsular bacteria before splenectomy.

The results of the phone follow-up with patients after hospitalization are presented in Table 21.1. The majority of patients did not make a decision about vaccination yet, and the minority were immunized. In general, for those who did not yet get vaccinated, the decision about the immunization was independent of the kind of pathogen of the three mentioned in the survey (Table 21.1; Fisher's Exact Test for Count Data, $p = 0.067$). Pairwise comparison of pathogens, however, shows a significant facilitating effect on that decision when *Streptococcus pneumoniae* and *Haemophilus influenzae* were mentioned ($p = 0.035$), as opposed to the other pairs of the pathogens, each involving *Neisseria meningitides*. This may be caused by *Neisseria meningitides* being less known as a pathogen by the lay public. The patients who already were immunized showed no preference for any of the three types of vaccinations ($Chi^2 = 3.6$, $df = 2$, $p = 0.165$).

4 Discussion

Vaccination coverage in our study was very low and adherence to Polish immunization guidelines was poor despite the fact that the splenectomy was performed as part of a scheduled procedure in most of the cases (61/85), so that the majority had an opportunity to be immunized before the procedure according to the existing recommendations. Nevertheless, most of the patients were neither immunized nor informed and their knowledge about immunizations other than hepatitis B, which is universally recommended before any surgical procedure, was highly unsatisfactory. That was the case regardless of the place of residency, so that access to health care services was not a factor.

The Netherlands has one of the highest immunization rates in splenectomized patients. In a retrospective study of 609 splenectomized patients from 28 hospitals there, 85.4 % of patients received a pneumococcal vaccine (vs. 20 % in our study), 39.4 % received immunization against *Haemophilus influenzae* b (vs. 9.4 % in our study) and 32.3 % against *N. meningitidis* group C (vs. 3.5 % in our study) probably thanks to recommendations from primary care physicians, mentioned in correspondence to the general practitioners in 80.5 % (vs. 23.5 % informed patients in our study) (Lammers et al. 2010).

In another study of Dutch patients treated in primary care who underwent splenectomy or had spleen dysfunctions, 56/130 (43.1 %) patients did not receive information concerning an increased

risk associated with functional or anatomical asplenia (vs. 69.4 % in our study) and 65/130 (50.0 %) patients did not know about the urgency of contacting their primary care physician in case of fever. Nevertheless, 103/130 (79.2 %) patients (vs. 20 % in our study) received the pneumococcal vaccination within a 5-year period. Vaccination rates against *H. influenzae* type b (42/130, 32.3 %) and *N. meningitidis* (35/130, 26.9 %) were much lower (Meerveld-Eggink et al. 2008), although higher than the respective 9.4 % and 3.5 %, in our study. In yet another retrospective Dutch study, 58 % of 95 splenectomized patients received prophylactic vaccinations (Brandenburg et al. 2008).

In a retrospective study by Fuentes-Ferrer et al. (2008), among 248 splenectomized Spanish patients (aged 61 on average), the immunization rate against *S. pneumoniae* was 48.4 %, and a statistically significant increase in the immunization rate was observed in the following years. In a study by Coignard-Biehler et al. (2011), among 92 adult French patients splenectomized between 2000 and 2005, the vaccination rates against *S. pneumoniae*, *H. influenzae* type, and *N. meningitidis* were 75, 37, and 10 % respectively. Before splenectomy, 15/92 adults were immunized against *H. influenza* type b and 5/92 against *N. meningitides* and after splenectomy these figures amounted to 20/92 and 2/92 patients, respectively. Only did 4 % of adult patients receive all the recommended vaccines. High vaccination rates for pneumococcal immunizations were achieved thanks to general practitioners.

The results of the studies cited above were presented by their authors as alarming, even though these results come out far better than those observed in our present study. It is important to note that the majority of splenectomized patients in our study were either elderly, being at risk of invasive pneumococcal disease due to age, potentially impaired immunity, and underlying chronic conditions, or young adults aged 21–30 years probably in contact with small children who often are carriers of respiratory tract pathogens, including *S. pneumoniae* and *N. meningitidis* (Carey and Pichichero 2004; Monto and Sullivan 1993).

The majority of splenectomized patients show an inadequate level of knowledge concerning this procedure or the consequences of the surgery (Corbett et al. 2007). Similarly, most of our respondents admitted that they did not realize the need for the recommended vaccinations before and after the splenectomy. It is worth noting that they were generally not informed either by primary care physicians or hospital surgeons about this issue. Providing this information is absolutely essential, since a doctor's recommendation is much more important in a patient's choice and decision regarding immunization than the price of the vaccine, which was clearly shown in our study as well as in the case of pneumococcal and influenza vaccinations (Johnson et al. 2008).

The main barriers to immunizations identified in the present study were lack of information and limited awareness of infectious dangers associated with asplenia as well as lack of clear advice from doctors involved in the management of splenectomy, since only informed patients were immunized (in 26/39 immunizations documented, in 5/39 declared but not documented; all patients informed by doctors were immunized). Other barriers were the price of vaccines, not reimbursed in Poland (in 5/8 informed, but not immunized patients), and misconceptions about the immunization (3/8 informed, but not immunized patients), whereas residency in rural areas was irrelevant concerning the rate of immunized patients. The barriers identified are similar to those found in the study by Johnson et al. (2008), which has consistently found that people fail to receive influenza vaccinations because they do not know they should be immunized. Most of the patients in our study were likely to follow their physician's recommendations for immunization, echoing earlier studies cited.

Although doctors remain the main source of knowledge about immunizations for patients in our study, most respondents rated information obtained as inadequate, and the transfer of the information as unconvincing. The patients

examined gained some information from Internet health portals, popular among the majority of chronically ill patients. However, this source of information cannot be considered sufficient, especially since some of our patients adopted misconceptions about immunizations from the Internet. Taking these observations into consideration, how can immunization rates be improved? Written protocols might be used by all doctors managing asplenic patients. On discharge from the hospital, a letter should be given to patients with information about immunization, antibiotic prophylaxis, and recommendations in case of fever. Splenectomized patients may also be referred to the general practitioner for information about infectious risks associated with asplenism (Coignard-Biehler et al. 2011).

The strength of our study lies in providing information about the adherence to current immunization guidelines in splenectomized patients in Poland, in having a nationally representative sample as the Institute of Hematology in Warsaw is the central institution in Poland where splenectomy is routinely performed, and in achieving a high 100 % response rate in the patients' surveys. The limitations, however, are a relatively small number of patients and that the barriers to immunization identified are only the most likely ones.

5 Conclusions

Patients splenectomized in Poland have one of the lowest immunization rates against capsular bacteria reported in literature and do not show an adequate level of knowledge concerning the consequences of the splenectomy. Primary care doctors and surgeons do not usually offer immunizations and essential information concerning the increased risk of life-threatening infections in splenectomized patients. The lack of conclusive information and orders from doctors as well as limited awareness of infectious dangers are the most likely barriers responsible for the low rate of immunization. It is important that all caregivers, surgeons, and primary care doctors give patients clear instructions and recommendations about immunizations and antibiotics recommended before and after splenectomy.

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

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Umbilical Cord Blood Gas Content, Postnatal State of Neonates, and Lactation After Caesarean and Natural Childbirth

22

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Abstract

In recent years the number of Caesarean sections (C-section) has been rapidly increasing. One of the reasons behind this is the fact that the scope of indications for these operations is still widening. The purpose of this article was to present a comparative analysis of the umbilical cord blood gas content, the state of infants, assessed by the Apgar score, and the course of lactation after elective C-sections or natural childbirth. We found that PO_2 in the cord blood after natural delivery was appreciably higher than that after C-section. The neonates delivered in a natural way also had an appreciably better Apgar score compared with those after C-section. Compared to mothers who delivered their babies in a natural way, it takes a longer time for C-sectioned women to commence breastfeeding. We conclude that a lower PO_2 level in the umbilical cord blood in women subjected to C-section may stem from breathing disorders in neonates at the time of delivery. The way of ending pregnancy has an apparent influence on adaptive abilities of infants to live outside mother's womb.

Keywords

Apgar score • Blood gases • Breathing • Childbirth • Lactation • Caesarean section • Umbilical cord blood

There are many factors that influence the condition of a baby after birth, such as mother's health, number of gametes that created an embryo, the process of the attachment of the fertilized ovum to endometrium, or mother's addictions and lifestyle. One can influence some of these factors by employing a number of diagnostic tests, specific therapy, or education of expectant mothers. The time and way of ending pregnancy is probably less appreciated as an element that also might influence the baby's condition.

The number of Caesarean sections (C-sections) is on the rise worldwide, reaching 15 % of all births globally and being as high as 21 % in developed countries (CDC NCHS 2010; Betrán et al. 2007). In the USA alone, the

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_22,

percentage of C-sections increased from 20.7 % in 1996 to 31.1 % in 2006 (MacDorman et al. 2008). This percentage may be even higher in teaching hospitals and reference centers due to cases of multiple pregnancies resulting from assisted reproductive techniques (Chong et al. 2012). There seems to be a consensus that the increase in C-section has to do more with changes in doctors' attitude and practice rather than with maternal requests and concern cases which do not necessarily bear an increased medical risk. The unchanging goal of the obstetrics is a natural delivery of a healthy baby. The greater the number of such deliveries, the higher is the assessment of the quality of medical services. For a baby, a natural delivery is an optimal way of leaving the uterine environment, as such delivery facilitates a newborn's adaptation to a life outside womb.

About 5 % of C-sections involve complications such as infant respiratory distress syndrome, tachypnea combined with wet lung syndrome, resulting from the retention of amniotic fluid in the respiratory tract, pulmonary hypertension, or injuries. There may also be problems with the postnatal adaptation, such as disturbed of thermoregulation, delayed development, or decreased muscle tone, one of the parameters of the Apgar score. Breathing problems after birth are usually temporary, but they have an impact on the Apgar score. C-section also may affect mother's lactation due to surgical stress, fatigue, pharmacological agents used in the process, and postponed attachment of a baby to the breast. The purpose of the present article was to compare the umbilical cord blood gas content, the postnatal state, and the course of lactation after elective C-sections with those in or natural childbirth delivery.

1 Methods

The study was approved by an institutional Research Review Board. The method consisted of a retrospective analysis of PO_2, PCO_2, and pH measurements of the umbilical cord blood and neonates' Apgar score after C-section or natural childbirth from the records of 100 neonates born by elective C-sections and 138 neonates born in a natural way during June–August of 2011 in the maternity ward of the Clinic of Gynecology and Obstetrics of the Medical University in Wroclaw, Poland. All infants weighed over 2.5 kg at birth.

The average age of patients giving birth in a natural way was 29 years, while that of C-section patients was 30 years. In case of natural deliveries, it was the second one for 35.5 %, the third for 8.7 %, the fourth for 2.2 %, the fifth for 0.7 %, and the seventh for 0.7 % mothers. For the C-sectioned mothers, the respective percentages were 53, 9, 1, and 2 %.

Apgar scores were evaluated in the first minute of life. The scoring system evaluates the neonate's condition, on an analog scale from zero to two, taking into account five parameters: skin color, heart rate, reflex responses, muscle tone, and respiratory effort. The results are interpreted in the following way: 10–9 – newborn in good condition, 8–7 – tired during delivery, 6–4 – moderate cyanosis, and 3–0 – severe cyanosis (Casy et al. 2001; Apgar 1953).

The course of lactation was assessed from the interviews in which the mothers were asked about the time of the first breast feeding, abundance of breast milk in the first 3 days of lactation, and the need to introduce an infant formula.

Data were given as means $\pm$ SD and were statistically compared with an unpaired *t*-test. $P < 0.05$ was taken as indicative of statistically significant differences.

2 Results

2.1 Baby Deliveries

Among the reasons underlying the decision for a C-section distinctly predominated a previously performed C-section in anamnesis (55 %), followed by ophthalmologic problems and fetuses' malpresentations, and then by twin and post-term pregnancies (Fig. 22.1). All these pregnancies ended up with the deliveries of infants in a good condition, without any dysfunctional features. The category of other indications

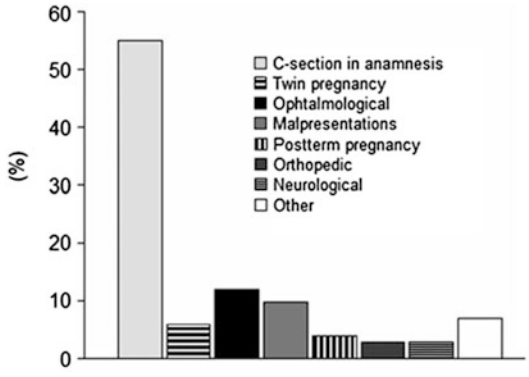

Fig. 22.1 Division of Caesarean sections depending on the underlying reason

Table 22.1 Apgar scores, umbilical cord blood gas content after C-sections and natural deliveries

	C-sections ($n = 100$)	Natural deliveries ($n = 138$)	P<
Apgar score	9.6 ± 0.8	9.9 ± 0.5	0.01
PO_2 (mmHg)	40.9 ± 16.9	46.7 ± 14.3	0.03
PCO_2 (mmHg)	41.3 ± 8.6	39.3 ± 9.6	NS
pH	7.35 ± 0.1	7.35 ± 0.1	NS

encompassed single cases of hematologic (1 %), laryngological (1 %), and psychiatric (1 %) pathologies, the wake of infertility treatments (1 %), suspected large fetuses (1 %) or feto-pelvic disproportion (1 %), and urinary incontinence (1 %).

Table 22.1 shows the mean values of the Apgar score and umbilical cord blood gas content in case of C-section and natural delivery. The cord blood PO_2 was significantly higher, on average, by about 6 mmHg, during natural deliveries compared with that during C-sections ($P < 0.03$), with otherwise normal cord blood gas content during both ways of child delivery. The higher PO_2 was associated with an appreciably better outcome of the Apgar score of the neonates, 9.6 ± 0.8 vs. 9.9 ± 0.5 in the neonates after C-sections and natural deliveries, respectively ($P < 0.01$), although the score denoted, on average, a very good condition of the babies born by both methods.

2.2 Course of Lactation

In the C-section group, 32 % of the mothers started breast-feeding in 2–4 h, 48 % in 5–8 h, and 20 % in more than 8 h after delivery. The respective times of the breast-feeding start after natural labor were 60, 28, and only 12 %. Fifty five percent of women after natural labor had an abundance of milk compared with 25 % of those after C-section. Forty two percent of infants after natural labor were given infant formula compared with 70 % of those delivered by a C-section.

3 Discussion

The finding of this study is that babies delivered in a natural way have an edge in the overall health status, as assessed by the Apgar score that includes the respiratory effort, over those delivered by a C-section, even though both types of deliveries end up in having healthy neonates whose basic features remain in the physiological range. A better status of neonates delivered by a C-section was associated with a greater PO_2 level in the umbilical cord blood. It is then a reasonable assumption that a higher PO_2 of the cord blood is influential in enhancing the neonates' health and the adaptive transition process from the *in utero* to external life. A healthy, born at term neonate, usually has a slight respiratory and metabolic acidosis immediately after birth due to the infusion of mother's lactic acid and periodic insufficient oxygenation during labor, with the possible PCO_2 level of up to 60 mmHg (Armstrong and Stenson 2007). However, when the child starts breathing regularly the acidosis withdraws, PO_2 increases, and PCO_2 decreases.

Infants delivered by C-sections, even those who are healthy and carried to term, are more prone to the repetitive fetal circulation and breathing disorders requiring intensive medical treatment. Gas exchange at the lungs may be hindered in these infants due to problems related to the absorption of fluid in the lungs,

dysfunctional surfactant, or a late first breath (Signore and Clebanoff 2008). Therefore, C-sections raise breathing problems considerably more often than those happening during natural deliveries and are linked to hypoxemia and respiratory distress syndrome in infants.

We further found that C-sections have an overall impeding effect on the course of lactation in that the breast-feeding is delayed, milk is less abundant, and babies are given infant formula more often compared with natural deliveries. These results are in accord with the observations of other authors (Wagner 2010).

Progresses in medicine, changes in obstetrics practice, and increasing safety of C-sections have all seen the rise in the number of women deciding to end their pregnancies in this way. However, Caesarean section still carries more risk than physiological delivery. The risk of patient's death in case of C-section is 3–10 times higher than that after physiological delivery. Harper et al. (2003) have shown that the risk of mother or infant death also is higher in case of elective C-sections. Nevertheless, indications for C-sections seem to increase and encompass not only an immediate threat to life and health of mother or child, or perinatal infections and complications, but also non-obstetrical reasons (Lavender et al. 2012). In the present study, the most frequent elective indications were a previous C-section, ophthalmologic indications, and malpresentations.

According to the recent recommendations of the European Association of Perinatal Medicine, C-sections due to medical indication after 39 weeks' gestation should be performed after the start of uterine contractions. On the other hand, elective C-sections should be conducted after the assessment of lung maturity and, in case of its shortage, after administering prenatal steroids, especially if there are indications for ending the pregnancy before 39 weeks. This also concerns elective C-sections performed as a protective measure after having had a previous C-section, being a frequent reason for this surgery (Tolloczko et al. 2010).

We conclude that C-sections have a negative influence on the adaptive capabilities of infants carried to term, especially on breathing disorders (lower Apgar scores and hypoxemia). C-sections also have a negative influence on the course of lactation in the first days following delivery (later occurrence of lactation, less milk, giving infant formula more frequently). An effort, therefore, should be undertaken to reduce the number of unnecessary C-sections, including pre-delivery parents education and counseling (Wiklund et al. 2012; Khunpradit et al. 2011).

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

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Respiratory Function in Pregnant Women

23

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Abstract

Respiratory disorders during pregnancy are connected with its physiology. About half of pregnant women suffer from dyspnea on exertion and some 20 % also from dyspnea at rest. Symptoms may intensify in obese patients. Smoking and respiratory disorders influence the well-being of the fetus. This study evaluates respiratory function in pregnant women as assessed by spirometry. The tests were carried out in 54 pregnant women in the 2nd and 3rd trimester. We found reduced values of vital capacity and expiratory reserve volume in all women, which suggests the existence a restrictive respiratory disorder in physiological pregnancy. Smoking seems to cause obstructive disorders; in smoking patients there was a reduction of the Tiffenau ratio. Participation in birth classes had a positive influence on inspiratory capacity. High BMI before pregnancy, excessive weight gain during pregnancy, or age of becoming pregnant did not appreciably influence spirometry results.

Keywords

Dyspnea • Spirometry • Respiratory function • Pregnancy • Women

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_23,

1 Introduction

Due to hormonal changes and increase in intra-abdominal volume, normal pregnancy may have a mechanical and functional impact on respiratory functioning. The percentage of pregnant women who complain of dyspnea varies from 50 % before the 20th week of pregnancy to 76 % past the 31st week of pregnancy (Milne et al. 1978). Hyperventilation observed in pregnant women is probably caused by the ventilatory stimulating effect of progesterone. Tidal volume (TV) and minute ventilation increase, while functional residual capacity (FRC) and expiratory reserve volume (ERV) decrease (Puranik et al. 1994; Contreras et al. 1991; Alaily and Carrol 1978). According to many authors, breath rate and forced expiratory volume in 1 s (FEV1) do not change (Grindheim et al. 2012; McAuliffe et al. 2002; Brancazio et al. 1997; Puranik et al. 1994; Das and Jana 1991). There have not been any definitive reports on changes in vital capacity (VC) during pregnancy.

Edema and hyperemia of the mucous membranes throughout the airways occur during pregnancy due to dilation of capillaries. Progesterone causes a widening of the trachea and bronchi, which contributes to increased alveolar ventilation. It has been found that FEV_1 during pregnancy is directly proportional to the infant's birth weight, but it is inversely proportional to intrauterine growth retardation, gestational hypertension, and preterm birth in asthmatic women (Schatz et al. 2006). Suboptimal respiratory system function may be expected in the course of uncomplicated pregnancy as well as in pregnant women who are pregestationally overweight, who experience excessive weight gain during pregnancy, or smoke cigarettes. Pregestational obesity and excessive weight gain during pregnancy are both recognized as being associated with increased risk of maternal complications during pregnancy (Dietl 2005; Rode et al. 2005; Castro and Avina 2002; Galtier-Dereure and Bringer 2002). It has been well documented that overweight individuals have a decrease in FEV_1 (Unterborn 2001). However, there are also reports showing no changes in FEV_1 in pregnant women who are pregestationally overweight or who gain excessive weight during pregnancy (Grindheim et al. 2012). It has been proven that cigarette smoking before pregnancy may have an adverse impact on the parameters for VC, FEV_1 or FEV_1/VC (forced expiratory volume in 1 s as % of vital capacity, Tiffenau index) (Das et al. 1991). An assessment of spirometry parameters during pregnancy in primigravidas, multigravidas, and women who attend birth classes could provide a valuable insight on this issue.

The goal of our study was to evaluate respiratory system functioning in pregnant women on the basis of spirometry tests and to determine whether active participation in birth classes and other factors such as pregestational obesity, excessive weight gain during pregnancy, cigarette smoking before pregnancy, gestational age, and the number of previous childbirths have an impact on respiration during pregnancy

2 Methods

2.1 Subjects

The study protocol was approved by the Ethics Committee of Wroclaw Medical University (reference No. KB–597/2012, dated July 17, 2012) and written informed consent was obtained from each patient.

Fifty four pregnant women were qualified for testing; 22 from birth classes and 32 hospitalized in high-risk pregnancy units who did not attend birth classes. Pregnant women screened for testing were in the 2nd or 3rd pregnancy trimester and did not have any accompanying conditions such as bronchial asthma, deformations of the chest (kyphosis, scoliosis), or catarrhal disorders of the upper airways. Pregnant women from birth classes were tested toward the course end. The women from the high-risk pregnancy units were admitted for a short stay due to the following reasons: to give birth, elective Caesarean,

glucose intolerance, gestational diabetic control, observation to exclude premature labor, or premature amniotic fluid leakage. None of the women had any contraindications for spirometry testing (e.g., amniotic fluid leakage, uterine activity, or the direct threat of preterm labor, and placenta praevia).

Thirty one (57.4 %) of all tested women complained of dyspnea. All women received a clinical questionnaire to fill in, asking for anthropometric parameters: data concerning their pregnancy, active participation in birth classes, medical history, stimulants, and respiratory problems during the current pregnancy. Spirometry and flow/volume tests were carried out with the use of a portable spirometer Lungtest 500 made by MES (Cracow, Poland), fitted with mouthpiece and pneumotachograph head. Each pregnant woman was tested once in the sitting position by members of the research team who had been trained in spirometer operation.

2.2 Spirometry

Spirometry test. With a nose clamp in place, participants were told to take ten slow breaths and were then instructed first to breathe out as deeply and slowly as possible and then to breath in as deeply and slowly as possible.

Flow/volume test. After taking a few slow breaths, the participant was instructed first to breathe out as deeply and slowly as possible, then to breathe in as quickly and intensively as possible, and then to breathe out as quickly and intensively as possible. This step was repeated several times.

The study also included an analysis of static parameters such as VC, ERV, and TV, and dynamic parameters such as FEV_1 and the Tiffenau index. Apart from the TV measured in liters, the remaining parameters are expressed as a percentage of the standard calculated for each tested pregnant woman.

2.3 Data Elaboration

Data are presented as means ± SD and minima-maxima. Since a Shapiro-Wilk rejected the hypothesis of normality of data distribution, a non-parametric Mann–Whitney U test was used to make comparisons between groups. A commercial Statistica 10.0 PL package was used for all quantitative analyses. $P < 0.05$ was considered significant.

3 Results

3.1 Baseline Characteristics

Baseline subjects' characteristics and demographic data are shown in Table 23.1. Of particular attention in the baseline spirometry results are lowered ERV, VC, and FEV_1 values, which indicate the presence of a respiratory disorder of a restrictive nature. The Tiffenau index was within normal ranges. Furthermore, increased TV (standard: 5–10 ml/kg) is also noteworthy in relation to body weight both before pregnancy and on the test day.

3.2 Birth Class Participants

Comparison of 22 pregnant women attending birth classes with the 32 women who did not attend the classes shows that there were no appreciable differences between the two groups concerning women's age, gestational age, height, or body weight before pregnancy, BMI before pregnancy, and weight gain during pregnancy. Table 23.2 compares the spirometry parameters in pregnant women attending vs. non-attending birth classes. There was a significant difference regarding TV in favor of the women attending birth classes. Of particular interest in both groups, although the differences were not statistically significant, are lowered VC and FEV_1 values, which indicate the presence of a mild respiratory disorder of a restrictive nature.

Table 23.1 Baseline characteristics and demographic data of all tested pregnant women (n = 54)

Age (year)	30.1 ± 3.6
	20–38
Gestational age (week)	33.1 ± 4.9
	21–42
Weight before pregnancy (kg)	65.0 ± 12.8
	45–100
Weight on the test day (kg)	76.2 ± 14.1
	53–111
Height (cm)	165.1 ± 6.4
	153–182
BMI before pregnancy (kg/m^2)	23.2 ± 4.1
	16–35
Weight gain during pregnancy (kg)	11.3 ± 5.9
	1–31
VC (% predicted)	88.5 ± 16.2
	59–126
ERV (% predicted)	67.6 ± 29.2
	26–146
TV (l)	0.84 ± 0.27
	0.36–1.54
FEV_1 (% predicted)	79.2 ± 18.0
	41–123
FEV_1/VC (% predicted)	92.9 ± 15.5
	49–129

BMI body mass index, *VC* vital capacity, *ERV* expiratory reserve volume, *TV* tidal volume, FEV_1 forced expiratory volume in 1 s, FEV_1/VC forced expiratory volume in 1 s as % of vital capacity; data are means ±SD and ranges.

Also, ERV values were low in both groups, especially in the group attending birth classes. The Tiffenau index in both groups was within the normal range.

3.3 High BMI Before Pregnancy

Data for 14 pregnant women with high BMI of 28.7 ± 2.9 kg/m^2 before pregnancy (H-BMI group) were separated from the test group and then compared with the group of 40 pregnant women with normal BMI of 21.3 ± 2.4 kg/m^2 before pregnancy (N-BMI group). There were the following significant differences between the N-BMI and H-BMI groups concerning body mass: before pregnancy (59.2 ± 7.7 vs. 81.6 ± 9.7 kg, p = 0.0001) and on the test day (70.5 ± 10.3 vs. 92.3 ± 10.9 kg, p = 0.0001). Table 23.2 shows the basic spirometry data comparing the N-BMI and H-BMI groups. There were no statistically significant differences between the groups. Lower ERV, VC, and FEV_1 values, pointing to the possibility of a mild respiratory disorder, were observed in both groups. In general, pregestational obesity was found not to have a worsening influence on spirometry parameters during pregnancy.

3.4 Excessive Weight Gain During Pregnancy

Thirteen pregnant women with excessive weight gain during pregnancy (H-PR group) were separated from the test group and compared to the group of 41 pregnant women with normal weight gain (N PR group). A normal weight gain during pregnancy was designated relative to the BMI before pregnancy. For a BMI < 18.5 kg/m^2, the normal weight gain was assumed to be 12.5–18.0 kg, for a BMI 18.5–24.9 kg/m^2 – 11.5–16.0 kg, for a BMI 25–29.9 kg/m^2 – 7.0–11.5 kg, and for a BMI ≥ 30 kg/m^2 – 5.0–9.0 kg. There were the following significant differences between the normal and excessive weight gain groups of women: pregnant women's age (29.6 ± 3.4 vs. 31.8 ± 3.9 years old, p = 0.034), gestational age (32.1 ± 4.9 vs. 36.3 ± 3.7 weeks, p = 0.005), body weight before pregnancy (61.7 ± 11.1 vs. 75.4 ± 12.8 kg, p = 0.001), body weight on the test day (70.8 ± 10.4 vs. 93.2 ± 10.3 kg, p = 0.0001), BMI before pregnancy (22.4 ± 3.6 vs. 25.7 ± 4.6 kg/m^2, p = 0.006), and weight gain during pregnancy (9.3 ± 3.7 vs. 17.4 ± 7.0 kg, p = 0.006). Table 23.3 shows the basic spirometry data comparing the normal and excessive weight gain groups of pregnant women. The results do not support the notion of an adverse effect of excessive weight gain during pregnancy on pulmonary function as assessed by spirometry. The VC value was even significantly higher in those women who had an excessive weight gain during pregnancy, as also the ERV, TV, and FEV_1 tended to be.

Table 23.2 Spirometry in pregnant women attending birth classes, non-attending birth classes, with normal <25 kg/m^2 BMI, (N-BMI group) and high >25 kg/m^2 BMI (H-BMI group)

	Birth class (n = 22)	Without birth class (n = 32)	N-BMI (n = 40)	H-BMI (n = 14)
VC (% pred)	88.8 ± 14.9	88.3 ± 17.2	87.9 ± 16.6	89.9 ± 15.3
	63.5–113.3	58.6–126.4	58.6–126.4	67.6–122.3
ERV (% pred)	57.5 ± 16.7	74.4 ± 34.0	67.6 ± 29.1	67.5 ± 30.9
	30.7–89.5	26.0–146.0	26.0–146.0	30.7–122.9
TV (% pred)	0.97 ± 0.30	0.75 ± 0.21**	0.83 ± 0.27	0.87 ± 0.30
	0.51–1.54	0.36–1.30	0.36–1.39	0.45–1.54
FEV_1 (% pred)	76.4 ± 15.6	81.1 ± 19.4	78.4 ± 19.1	81.4 ± 14.7
	48.8–105.7	41.2–123.3	41.2–123.3	49.0–105.7
FEV_1/VC (% pred)	88.9 ± 16.0	95.7 ± 14.7*	92.2 ± 15.0	94.8 ± 17.0
	49.2–129.1	53.5–116.5	53.5–129.1	49.2–116.5

See Table 23.1 for spirometric acronyms; data are means ±SD and ranges.
*$p < 0.05$ and **$p < 0.01$ vs. birth class

Table 23.3 Spirometry in pregnant women with normal and excessive body weight gain, and in groups of non-smoking and smoking before pregnancy

	Normal weight gain (n = 41)	Excessive weight gain (n = 13)	Non-smoking (n = 45)	Smoking prepregnancy (n = 9)
VC (% pred)	85. 3 ± 15.5	98.9 ± 14.4*	89.2 ± 15.5	85.0 ± 19.8
	58.6–113.6	79.0–126.4	58.6–126.4	63.5–113.6
ERV (% pred)	65.0 ± 28.6	76.1 ± 31.0	65.9 ± 27.4	75.9 ± 38.1
	26.0–146	32.0–122.9	26.0–126.1	32.0–146
TV (% pred)	0.82 ± 0.27	0.92 ± 0.31	0.87 ± 0.27	0.73 ± 0.27
	0.36–1.39	0.61–1.54	0.450–1.540	0.30–1.30
FEV_1 (% pred)	77.9 ± 16.7	83.4 ± 21.6	81.3 ± 16.2	68.9 ± 23.7
	44.0–123.3	41.–116.5	41.2–123.3	44.0–23.7
FEV_1/VC (% pred)	94.9 ± 14.1	86.3 ± 18.6	95.0 ± 14.4	83.1 ± 17.4*
	70.9–129.1	49.2–108.0	53.5–129.1	49.2–104.0

See Table 23.1 for spirometric acronyms
*$p < 0.05$ vs. normal weight gain and vs. non-smoking

3.5 Cigarette Smoking Before Pregnancy

Nine pregnant women who said they smoked cigarettes before pregnancy were separated from the test group and then compared with the group of 45 non-smoking pregnant women. There were no significant differences between the two groups concerning women's age, gestational age, body weight before pregnancy, height, BMI before pregnancy, or weight gain during pregnancy. Table 23.3 shows the basic spirometry data comparing the non-smoking and smoking groups. The Tiffenau index and FEV_1 appeared worse in those who smoked before pregnancy, although the latter did not assume statistical significance. The results suggest that smoking before pregnancy may facilitate an overlap of airway obstruction on a restrictive pattern of airway disorder; the latter being more typical during pregnancy.

Table 23.4 Spirometry in pregnant women at 20–36 weeks of gestation (20–36 HBD) and 37–42 weeks of gestation (37–42 HBD) as well as in primigravidas (PG) and multigravidas (MG)

	20–36 HBD (n = 37)	37–42 HBD (n = 17)	PG (n = 39)	MG (n = 14)
VC (% pred)	89.0 ± 16.7	88.3 ± 17.2	86.5 ± 14.1	95.0 ± 20.0
	58.6–122.3	58.6–126.4	63.5–113.3	58.6–126.4
ERV (% pred)	66.5 ± 30.0	74.4 ± 34.0	62.7 ± 24.4	81.9 ± 36.7
	26.0–146.0	26.0–146.0	26.0–126.1	33.0–146.0
TV (% pred)	0.85 ± 0.30	0.75 ± 0.21	0.86 ± 0.29	0.79 ± 0.22
	0.36–1.54	0.36–1.30	0.36–1.54	0.49–1.30
FEV_1 (% pred)	78.4 ± 17.8	81.1 ± 19.4	75.2 ± 16.0	91.7 ± 17.9*
	44.0–123.3	41.2–123.3	41.2–105.7	57.3–123.3
FEV_1/VC (% pred)	92.0 ± 15.0	95.7 ± 14.7	90.3 ± 16.0	100.6 ± 11.8*
	49.2–116.5	53.5–116.5	49.2–129.1	76.7–116.5

See Table 23.1 for spirometric acronyms; data are means ±SD and ranges.
*$p < 0.05$ vs. primigravidas

3.6 Gestational Age

To analyze whether gestational age affects the occurrence of respiratory disorders, all tested pregnant women were divided into 2 groups: low gestational age (20–36 weeks HBD; 37 women) and high gestational age (37–42 weeks HBD; 17 women). The only significant difference between the respective groups concerned the weight gain during pregnancy (9.4 ± 3.8 vs. 15.9 ± 7.6 kg, p = 0.001). Table 23.4 shows the basic spirometry data comparing the 20–36 HBD and 37–42 HBD groups. There were no statistically significant differences between the two groups, which implies that gestational age had no appreciable influence on pulmonary function; although somewhat lower VC, ERV, and FEV_1 values were observed in both groups.

3.7 Primigravidas and Multigravidas

Thirty nine primigravidas (PG group) were separated from the test group and then compared with the group of 14 multigravidas (MG group). The group differed concerning the women's age; the PG being younger (29.3 ± 3.1 vs. 32.6 ± 3.8 years old, respectively, p = 0.005). Table 23.4 shows the basic spirometry data comparing the PG and MG groups. Significant differences in favor of multigravidas were noted in the FEV_1 and Tiffenau index. The VC and ERV values also were higher in the multigravidas, although the difference failed to assume statistical significance. The results suggest that respiratory function may actually improve during subsequent pregnancies.

4 Discussion

The present study assessed respiratory function in pregnant uncomplicated pregnancy based on spirometry. Apart from pregnancy, we took into account the possible influence on respiratory function of a number of other accompanying factors such as body weight, cigarette smoking, and participation in birth classes. The percentage of pregnant women complaining of dyspnea in our study amounted to 57.4 %, with an average gestational age of 31.1 weeks (Table 23.1). That figure is lower than data provided by Milne et al. (1978) who noted a reported feeling of dyspnea in 76 % of pregnant women with gestational age of more than 31 weeks, with a rising tendency thereafter. The occurrence of dyspnea before the 20th week of pregnancy is thought to manifest hormonal rather than mechanical reasons (Milne et al. 1978; Milne 1979). Research conducted in the 1950s and 1960s showed that progesterone administered to volunteers increases minute

ventilation, which lowers the alveolar air PCO_2 (Tyler 1960; Goodland et al. 1953).

In the present study, a relatively low ERV value was observed in all studied groups of pregnant women. This is in accordance with the reports by other authors (Puranik et al. 1994; Contreras et al. 1991; Alaily and Carrol 1978). In addition, we also observed lowered VC values. Previous studies on VC changes during pregnancy have not been conclusive; reporting lack of change and an increase or decrease in VC (Milne 1979; Alaily and Carrol 1978). Overall, spirometric changes we observed are commensurate with a mild restrictive pattern, which seems associated with normal pregnancy. A lowered FEV_1 value was observed in the majority of pregnant women, which, on the other side, may suggest a mild obstructive pattern. Other authors have reported no change in FEV_1 (Grindheim et al. 2012; McAuliffe et al. 2002; Brancazio et al. 1997; Puranik et al. 1994; Das and Jana 1991). In the present study, the majority of women had the Tiffenau index in a normal range, which may reflect a bronchodilatory effect of progesterone. In turn, TV was higher than normal, which is in accordance with other observations authors (Puranik et al. 1994; Contreras et al. 1991; Alaily and Carrol 1978). Neither were high BMI before pregnancy, excessive weight gain during pregnancy, or gestational age found to worsen spirometry parameters, especially the FEV_1. That is in accordance with earlier reports (Grindheim et al. 2012; Unterborn 2001; Das et al. 1991). A significant worsening of the Tiffenau index and a lower FEV_1 value were found in the group of pregnant women who smoked before pregnancy; pointing to the possibility of obstructive disorders, apart from the restrictive ones (Das et al. 1991). That suggest that the bronchodilatory effect normally expected in pregnancy is insufficient to overcome the deleterious effects of cigarette smoking. In the present study we also report that active participation in birth classes had an enhancing effect on TV and that pulmonary function has an improving tendency with the number of subsequent pregnancies in multigravidas.

In conclusion, the present study demonstrates that more than half of pregnant women complain of dyspnea which is not really reflected in pulmonary function deterioration as assessed by spirometry. The predominant spirometric change is one of a mild airway restriction. Mechanical and psychophysiological factors accompanying pregnancy should also be taken into account as possible contributors to the feeling of dyspnea.

Acknowledgements We would like to thank the staff of the birth classes of the University Hospital No 1 in Wroclaw and the birth classes in the Wroclaw Informative-Educational Centers for their kindness and help during the study.

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

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Lung Function in Patients with Gastro-Esophageal Reflux Disease and Respiratory Symptoms

24

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Abstract

The aim of this study was to investigate lung function in patients with gastro-esophageal reflux disease (GERD) who present respiratory symptoms suggestive of the possibility of co-morbid asthma. The study encompassed 20 patients (9 women and 11 men; age range from 11 to 68 years) diagnosed with GERD and presenting with chronic cough and other non-specific periodic respiratory complaints. The control group consisted of closely gender and age-matched 20 subjects without any gastrointestinal or respiratory symptoms. All patients and control subjects were tested for lung function, which encompassed spirometric and flow-volume variables. We found that none of the GERD patients had lung function abnormalities characteristic of asthma. There were, however, decreases in forced expired volume in 1 s, forced vital capacity, and in maximal instantaneous forced expiratory flows in the GERD patients compared with the healthy subjects. We conclude that cough accompanying GERD is unlikely to be associated with the presence of co-morbid asthma, but rather suggests a mild airway inflammation developing as a sequel of GERD. The corollary is that chronic cough should prompt physician's attention to consider diagnostic work-up toward the possibility of GERD.

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_24,

Keywords
Acid aspiration • Asthma • Airway obstruction • Gastric content • Gastro-esophageal reflux disease (GERD) • Lung function • Spirometry

1 Introduction

Gastro-esophageal reflux disease (GERD) afflicts approximately 7–10 % of the general public, particularly in highly developed societies. The disease may present symptoms every day, and about 20 % of afflicted persons develop symptoms at least once a week. The risk factors for GERD encompass obesity, pregnancy, alcohol abuse, smoking cigarettes, a diet rich in chocolate, tomatoes, and coffee, oral contraception, and beta2 adrenergic receptor agonists (Boguradzka et al. 2006). Kling (2012) has reported that GERD prevalence is higher in children with neurological disorders, chronic lung disease, and prematurity. The symptoms are usually evoked by the backflow of stomach acids into the lower part of esophagus due to the lower esophageal sphincter (LES) insufficiency or hiatal hernia (D'Urbano 2012). GERD typically runs such symptoms as heartburn, regurgitations, dysphagia, or teeth erosions. However, a variety of atypical symptoms may also manifest, which include hoarseness, chest pain, dry cough, or airway inflammatory reactions. Dry paroxysmal cough and airway inflammation can be easily taken for asthma. In fact, a distinct type of asthma, called gastro-esophageal reflux asthma, is distinguished by some clinicians.

Mutual interaction between GERD and asthma or asthma-like syndromes remains of high clinical interest, albeit the cause-effect relationship has never been conclusively resolved, despite extensive research to this end (Pirogowicz et al. 2010). The prevalence of GERD among the asthmatic population is reportedly higher than that in the general population; the range varies greatly from about 20–30 to 90 % depending on the study population (see Gaude and Karanji 2012; Kling 2012 for reviews). The spread of the incidence rate above outlined underscores the indecisiveness of the issue. The major problem is of whether asthma triggers GERD or it is the other way around. In the present study we reasoned that if asthma would be antecedent to GERD, then the subjects suffering from GERD, particularly those presenting paroxysmal, chronic cough, ought to demonstrate the characteristic of asthma impairment in lung function of the obstructive type. The objective of the study was, therefore, to assess lung function in subjects who were diagnosed with GERD.

2 Methods

The study was approved by a local Ethics Committee and the participating subjects gave informed consent for the study procedures. There were two groups of subjects in this study. One group consisted of patients who presented with paroxysmal cough that was accompanied by heartburn, regurgitations, hoarseness, and other non-specific symptoms. These patients were diagnosed with GERD, confirmed with pH-metry and gastro-endoscopic examinations. There were 20 patients in this group (F/M; 9/11), ranging in age from 11 to 68 years. The control group consisted of another 20 subjects who were gender and age-matched and were in good general state of health, with neither asthma nor GERD in anamnesis. In all subjects of both groups lung function tests consisting of spirometry and flow-volume measurements were carried out, with Lungtest 1,000 equipment (MES, Cracow, Poland). Spirometry results expressed in litres were converted to % predicted according to the European Community for Steel and Coal standards (Quanjer et al. 1993). Data were

presented as mean values ± SD. The normality of distribution was tested with a Shapiro-Wilk test, and then a parametric *t*-test or non-parametric Mann–Whitney test was used, as required, to compare intergroup differences. A $P < 0.05$ was taken to indicate statistically significant differences. A commercial statistical package, Statistica 10, was used for all analyses.

3 Results and Discussion

There was no significant difference between the GERD and control groups in the majority of spirometric variables. The only significant difference between the two groups was modest decreases in FEV1, FVC, MEF50%, and MEF25% ($p < 0.05$) in the GERD group, although all these induces remained within the acceptable range (Table 24.1). MEF measurements were characterized by relatively high standard deviations, which indicate their low reproducibility. As instantaneous MEF measurements are derived from the flow-volume curve, they are known to be more sensitive to small and meaningless random flow oscillations, which may explain their erratic reproducibility. On the other side, every disorder that affects FVC will also influence MEFx%. Since during a forced expiration, exhaled volume does not increase linearly with time, in case of the obstructive pattern, like that in asthma, and reduced FVC, the time required to expire to MEFx% level, and thus the severity of obstruction, may be underestimated. Although the predictive value of MEFx% is limited, their decrease may speak to a developing tendency for obstruction, which may reflect, e.g., an ongoing inflammatory alterations in small bronchi. The source of this inflammation cannot be established by functional lung tests performed, but it might be related to the underlying GERD. However, we failed to demonstrate typical for full-fledged or persisting asthma changes in lung function of the obstructive type.

The finding of this study was that patients suffering from GERD and simultaneously presenting with chronic paroxysmal cough and other nonspecific airway symptoms, and thus suspected of having an asthmatic co-morbidity, did not have changes in lung function characteristic of asthma. In the group of 20 GERD patients, there was not a single case in which the existence of asthma-like obstructive changes could be substantiated in the measurement of lung function. Therefore, it is unlikely that asthma was antecedent to GERD and could promote the development of GERD. Likewise, it is unlikely that the persisting GERD led to the development of asthma. Nevertheless, we found some modest unspecific changes in lung function, particularly concerning small bronchial, which could indicate an ongoing inflammatory process in the airways during GERD. Thus, GERD may enhance susceptibility to airway inflammation in the long-run.

3.1 Gastro-Esophageal Reflux as Trigger of Bronchial Asthma

There are two potential mechanisms which could explain how the backflow of stomach acid could produce bronchoconstriction and evoke asthma-like symptoms.

3.1.1 Microaspiration of Acid and Bronchospastic Response

The bronchospastic response may underlie reflux-associated asthma reported in some studies. In the cat model, intratracheal acidification with 0.2 ml hydrochloric acid results in a 4.6-fold increase in the mean inspiratory and expiratory times and increases total lung resistance (Ownby et al. 2002; Tuchman et al. 1984). Donnelly et al. (1993) demonstrated in humans during simultaneous measurements of intraesophageal and intratracheal pH that acidic pH lower than 5.0 coincided with esophageal pH lower than 4.0. A considerable improvement in the tracheal pH, along with that in the patients' condition was observed after anti-reflux surgery. These findings were confirmed in another study in which there were 129 patients with the predominantly

Table 24.1 Lung function tests in the gastro-esophageal reflux disease (GERD) patients and healthy subjects

	FEV1	FVC	PEF	MEF75	MEF50	MEF25	VC	FEV1%FVC
GERD ($n = 20$)	96.3 ± 12.5*	105.1 ± 11.4*	100.6 ± 16.2	92.2 ± 25.1	83.5 ± 34.8*	70.7 ± 38.7*	102.0 ± 10.1	95.0 ± 10.9
Controls ($n = 20$)	109.0 ± 12.7	113.6 ± 12.0	99.6 ± 13.6	103.6 ± 14.9	116.0 ± 24.5	118.2 ± 36.5	108.9 ± 12.5	101.4 ± 10.3

Data are means ± SD %predicted
FEV_1 forced expiratory volume in 1 s, *FVC* forced vital capacity, *PEF* peak expiratory flow, *MEF75* maximal instantaneous forced expiratory flow where 70 % of the FVC remains to be expired, *MEF50* maximal instantaneous forced expiratory flow where 50 % of the FVC remains to be expired, *MEF25* maximal instantaneous forced expiratory flow where 50 % of the FVC remains to be expired, $FEV_1\%FVC$ Tiffenau index
*$P < 0.05$

respiratory symptoms out of the 300 patients with gastro-esophageal reflux. In 96 out of the 129 improvement was noted after surgical correction of the reflux (Lomasney 1977). Microaspirations, assessed from a decrease in intratracheal pH at the time of a reflux episode, appear to take place in about 20 % of severe asthma patients (Sontag et al. 1990; Jack et al. 1995; Donnelly et al. 1993). Studies report that as much as 80 % of asthmatic patients have significant gastro-esophageal reflux on 24-h pH monitoring, most likely related to microaspirations. However, the issue of mutual interaction between microaspirations and asthma symptoms is still unsettled. Gustafsson et al. (1990), for instance, have demontrated just a few episodes of reflux in children with asthma and concluded that acid aspiration is rather an unlikely trigger of asthma.

3.1.2 Reflex Bronchoconstriction – Vagally Mediated Reflex

Stimulation of esophageal acid sensitive receptors initiates afferent signals in the vagus nerves. This leads to a reflex constriction not only of esophageal smooth muscles but also airway smooth muscles. This interaction is explained as a consequence of common innervation (Gaude and Karanji 2012). Donnelly et al. (1993) performed a double-blind acid infusion in four groups of subjects: normal controls, patients with asthma and GERD, patients with asthma without GERD, and patients with GERD without asthma. Seventy two percent of patients with GERD and asthma presented increased respiratory resistance, compared with just 10 % of patients with asthma alone.

3.2 Bronchial Asthma as Trigger of Gastro-Esophageal Reflux

Many authors suggest the converse correlation of asthma as a possible trigger of reflux esophagitis. There also are two potential mechanisms which could underlie this interaction.

3.2.1 Asthma Medications

Anti-asthmatic therapy such as systemic beta2-adrenergic receptor agonists or theophylline may lead to a general relaxation of smooth muscles, including the lower esophageal sphincter (LES), and thus may decrease LES pressure and facilitate the appearance of GERD symptoms. This mechanism of asthma treatment-related reflux has been refuted in a study of Michoud et al. (1991) who showed that the beta2-agonist salbutamol in a dose of 4 mg *per os* fails to affect the LES pressure gradient, esophageal contraction pressure, or the severity of reflux episodes either in asthmatics or in healthy subjects. Moreover, the authors showed that in fact asthmatics have a higher resting LES pressure than that in healthy subjects.

In a study of Zerbib et al. (2002) the effects of bronchial obstruction on LES motility and reflux have been reinvestigated. The study investigated LES motility and esophageal pH after methacholine-induced bronchospasm and after inhalation of salbutamol. In asthmatics, as opposed to healthy subjects, methacholine-induced decrease in FEV1 was accompanied by a transient increase in LES relaxation and in reflux episodes. Inhalation of salbutamol counteracted LES relaxation, but did not affect the number of reflux episodes. The authors conclude that bronchoconstriction may have to do with gastro-esophageal reflux, although the mechanisms of the interaction between the two phenomena remain enigmatic. At any rate, both studies above outlined refute the notion of a relaxing influence on LES of beta2-agonists commonly used in asthma treatment as the underlying cause of the gastro-esophageal reflux.

3.2.2 Mechanical Causes

It has been suggested that an obstructive pattern of asthma, raising negative pleural pressure, may increase the transdiaphragmatic pressure gradient, which may facilitate the backflow of gastric content into the esophagus. That, in turn, would set LES at a mechanical disadvantage as its function is boosted by the crural portion of the diaphragm (reviewed in Gaude and Karanji 2012).

4 Conclusions

In a group of patients suffering from gastro-esophageal reflux disease (GERD), presenting with chronic paroxysmal cough as the predominant clinical symptom, we failed to substantiate the co-existence of asthma, as an accompanying pathological condition, in any of the 20 patients studied. Lung function measurements provided no confirmatory results of the obstructive pattern characteristic of asthma, although some nonspecific alterations in maximal instantaneous forced expiratory flow found could point to increased proinflammatory readiness of airways. The study, therefore, provides no supportive evidence for a close interrelationship between GERD and asthma, and the often mutual appearance of both conditions. We submit, however, in line with some other authors (Irwin et al. 1993), that chronic unexplained cough should prompt attention to the diagnostic work-up of GERD as cough might be its presenting manifestation due rather to irritation of the esophagus than intratracheal aspiration of gastric content.

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

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Efficacy of Noninvasive Mechanical Ventilation in Obese Patients with Chronic Respiratory Failure

25

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Abstract

Chronic respiratory failure (CRF) develops in a minority of obese patients. Noninvasive mechanical ventilation (NIMV) is a new optional treatment for such patients. The aim of this study was to evaluate the effectiveness of NIMV in obese patients with CRF. The material of the study consisted of 34 obese patients (body mass index 47.3 ± 7.9 kg/m^2) with CRF (PaO_2 = 6.40 ± 0.93 kPa and $PaCO_2$ = 8.67 ± 2.13 kPa) who were hypoxemic despite an optimal therapy. Thirteen patients had an overlap syndrome (OS) – chronic obstructive pulmonary disease (COPD) coexisting with obstructive sleep apnea syndrome (OSAS) and 21 patients had obesity-hypoventilation syndrome (OHS). Ventilation parameters were determined during polysomnography. The efficacy of NIMV was evaluated on the fifth day and after 1 year's home treatment. We observed a significant increase in the mean blood oxygen saturation during sleep in all patients; the increase was greater in patients with OHS (92.6 ± 1.4 %) than in patients with OS (90.4 ± 1.8 %). There was a significant improvement of diurnal PaO_2 and $PaCO_2$ on the fifth day of NIMV (mean PaO_2 increase 2.1 kPa and $PaCO_2$ decrease 0.9 kPa) and also after 1 year of home NIMV (mean PaO_2 increase 1.9 kPa and $PaCO_2$ decrease 2.4 kPa). Only one patient stopped treatment because of lack of tolerance during the observation period (1–3 years). In conclusion, NIMV is an effective and well tolerated treatment option in obese patients with CRF resulting in a rapid relief of respiratory disorders during sleep and a gradual, long-term improvement of gas exchange during the day, particularly in patients with OHS.

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_25,

Keywords
Chronic respiratory failure • Chronic obstructive pulmonary disease (COPD) • Noninvasive mechanical ventilation (NIMV) • Obesity • Obesity-hypoventilation syndrome (OHS) • Obstructive sleep apnea syndrome • Overlap syndrome • Polysomnography

1 Introduction

Obesity is a social problem, reported globally. Current data suggests that the amount of persons in the highest body mass index (BMI) groups (>40 kg/m^2) is increasing at rates two and three times faster than those with a BMI of 30 kg/m^2 (Sturm 2007). Obesity affects significantly the respiratory system, decreasing lung volumes, increasing work of breathing, affecting respiratory muscle function and ventilatory control (Steier et al. 2009). Most of the obese are able to maintain awake eucapnia, but some of them develop chronic respiratory failure (CRF). It is usually due to the chronic alveolar hypoventilation that causes an increase of arterial partial pressure of carbon dioxide ($PaCO_2$) above 6 kPa. This is usually a consequence of obstructive sleep apnea syndrome (OSAS) and/or obesity-hypoventilation syndrome (OHS) (Borel et al. 2011; Leech et al. 1991).

Noninvasive mechanical ventilation (NIMV) used for home mechanical ventilation is a well-established and increasingly used therapeutic option for patients with chronic hypercapnic respiratory failure due to chronic obstructive pulmonary disease (COPD), neuromuscular or rib cage diseases (Lloyd-Owen et al. 2005; Simonds 2003; Mehta and Hill 2001; American College of Chest Physicians 1999). There are few data presenting the effects of NIMV therapy in severe obese patients with CRF especially in a long period of time (Perez de Llano et al. 2005; Janssens et al. 2003). Therefore, the aim of this study was to evaluate the effectiveness of NIMV in obese patients with chronic alveolar hypoventilation in a short and long period of time.

2 Methods

The study was approved by the Ethics Committee of Wroclaw Medical University and written informed consent was obtained from all study patients.

2.1 Patients and Diagnoses

The studied group consisted of 34 severely obese patients of the mean age 55 $\pm$ 11 years and BMI 47.3 $\pm$ 7.9 kg/m^2 (F/M 11/23) admitted to the Department of Pulmonology and Lung Cancer, Medical University of Wroclaw in a period of 2008–2012. The patients were assessed either for stable chronic respiratory failure or treated following an episode of acute decompensated respiratory failure. Study inclusion criteria were BMI $>$ 30 kg/m^2; daytime stable respiratory failure with $PaCO_2 > 6$ kPa; hypoxemia despite optimal therapy, including oxygen therapy or CPAP (continuous positive airway pressure). The exclusion criterion was an inability to provide written informed consent.

The final diagnoses of the studied 34 individuals are shown in Fig. 25.1. We diagnosed COPD with OSAS in 13 patients (overlap syndrome-OS) and OHS in 21 patients. In the group of OHS patients, 14 of them had coexisting OSAS (OHS + OSA). According to Mokhlesi (2010) OHS was defined as a combination of obesity (BMI $\geq$ 30 kg/m^2), daytime hypercapnia ($PCO_2 \geq 6$ kPa), and various types of sleep-disordered breathing after ruling out other disorders that may cause alveolar hypoventilation, such as like severe restrictive, obstructive

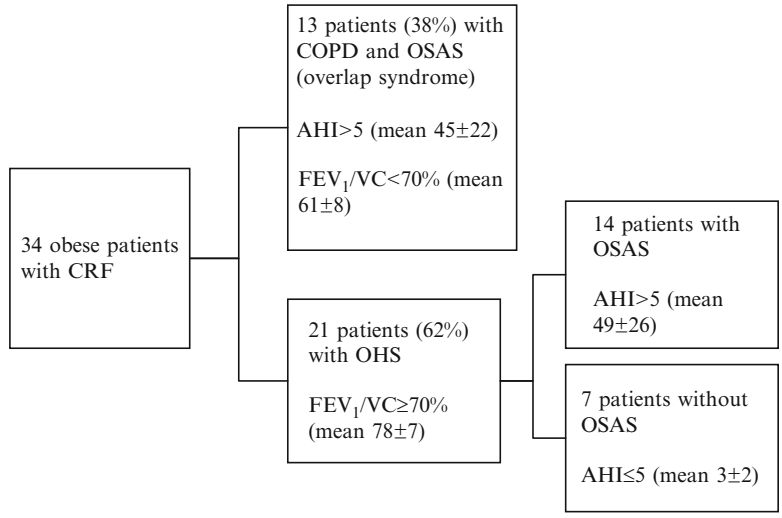

Fig. 25.1 Final diagnosis as a reason of chronic respiratory failure in the studied population, based on polysomnography and spirometry. *CRF* chronic respiratory failure, *COPD* chronic obstructive pulmonary disease, *OS* overlap syndrome, *OHS* obesity hypoventilation syndrome, *AHI* apnea/hypopnea index, *FVC* forced vital capacity, FEV_1 forced expiratory volume in 1 s. Values are means ± SD

pulmonary diseases, chest wall disorders, severe hypothyroidism, or neuromuscular diseases. COPD and OSA were diagnosed according to the following guidelines: Global Strategy for Diagnosis, Management, and Prevention of COPD (2011) and The Report of an American Academy of Sleep Medicine Task Force (1999).

2.2 Study Protocol

All patients underwent baseline assessments of spirometry, arterial blood gas measurement, full polysomnography (PSG) before treatment, on the fifth day of NIMV therapy and during follow-up visit 1 year later. The PSG was performed with an Aura setup (Grass Technologies; West Warwick, RI) and spirometry with a Lung test 1,000 system (MES, Krakow, Poland) according to guidelines recommended by Quanjer and the European Respiratory Society (1993). NIMV parameters were established during first night under PSG control to avoid apneas and snoring and to achieve the adequate nocturnal respiratory control. Supplementary oxygen was provided to patients who were hypoxemic despite NIMV treatment ($PaO_2 < 7.3$ kPa). After establishing parameters of NIMV and achieving an improvement, the patients were discharged from the hospital and followed up at 12 months.

Ventilation parameters were determined during the polysomnography under the medical supervision. Most of the subjects (83 %) received NIMV via a Trilogy ventilator (Philips-Respironics, USA) in a pressure support, spontaneous/timed mode (PS-S/T). The patients used the optional AVAPS (average volume assured pressure support) mode that automatically adapts pressure support – inspiratory positive airway pressure (IPAP) to provide the preset patient's average tidal volume (VT). In that mode, IPAP was than titrated during ventilation in steps of 1 mbar/min to achieve a desired VT In the present study, the IPAP was set between expiratory positive airway pressure (EPAP) and 30 mbar. AVAPS was set to 7–10 ml/kg of ideal body weight. Ventilator settings were changed according to the patient's daytime and nocturnal tolerance, and to the maximal decrease of $PaCO_2$. During 1 year's treatment, the settings

Table 25.1 NIMV parameters

Variables	
IPAP (mbar)	19.2 ± 4.0
EPAP (mbar)	8.5 ± 3.0
VT (ml)	583.2 ± 68.1
Vleak (ml)	55.1 ± 14.1
f (breaths/min)	16.5 ± 3.1
PS-S/T AVAPS mode (% of patients)	84.1 ± 7.2
Oxygen (l/min)	2.6 ± 0.9
Patient-triggered breaths (%)	45 ± 27
Compliance (h:min/day)	5:23 ± 02:45
Supplemental oxygen therapy (n)	26

Values are means ± SD
IPAP inspiratory positive airway pressure, *EPAP* expiratory positive airway pressure, *VT* tidal volume, *Vleak* leakage volume, *f* breathing frequency, *PS-S/T* pressure support-spontaneous/timed mode, *AVAPS* Average volume assured pressure support mode

for EPAP, respiratory rate, and inspiratory/expiratory ratio were kept at the same level. Twenty six received supplemental oxygen (Table 25.1).

The differences between groups were assessed by a paired *t*-test. Statistical significance was assumed with at a $p < 0.05$.

3 Results

In the group of 34 patients we diagnosed the OS coexisting with of COPD and OSAS in 13 patients and OHS in 21 patients (Fig. 25.1). The analysis of different clinical variables of both groups (OS and OHS) showed a significantly lower FEV_1/VC ratio in the OS patients (65 ± 8 % vs. 78 ± 7 %, $p < 0.05$). The patients with both OS and OHS suffered from severe sleep apnea syndrome with similar high apnea/hypopnea (45.0 ± 21.1 vs. 47.9 ± 28.1, $p > 0.05$) and desaturation indexes (67.2 ± 25.6 vs. 64.3 ± 24.8, $p > 0.05$) (Table 25.2).

After the initiation of NIMV we observed an improvement of the clinical status of the patients soon after the onset of treatment and 1 year afterward. On the fifth day of NIMV, there was an increase in the mean SaO_2 during sleep in all patients, but it was greater in the patients with obesity-hypoventilation syndrome (92.6 ± 1.4 %) than in those with the overlap syndrome (90.4 ± 1.8 %, $p < 0.05$). We observed a significant improvement of diurnal PaO_2 and $PaCO_2$ soon after the beginning of NIMV (mean PaO_2 increase of 2.1 kPa and $PaCO_2$ decrease of 0.9 kPa on the fifth day of NIMV) and then after a year's home NIMV (mean PaO_2 increase of 1.9 kPa and $PaCO_2$ decrease of 2.4 kPa) (Table 25.3). During the observation period, two patients died (one patient with the overlap syndrome and one with the obesity-hypoventilation syndrome coexisting with OSA), and one patient stopped treatment because of lack of tolerance.

After 12 months' NIMV treatment we did not observe any significant changes in spirometry results. There was a small decrease of BMI after 12 months of NIMV (before: 47.3 ± 7.9 kg/m^2 and after: 44.7 ± 6.6 kg/m^2, $p > 0.05$), but it did not reach statistical significance (Table 25.4).

4 Discussion

The prevalence of respiratory failure is estimated between 20 and 30 % in hospitalized obese adult patients (Borel et al. 2011). The presence of hypercapnia and hypoxemia in patients with severe obesity is a consequence of complex interactions between a lot of factors associated with obesity itself, respiratory drive and sleep disordered breathing and in some cases coexisting chronic lung disease (Piper and Grunstein 2010). One of the most common reasons of respiratory failure in obesity is the obesity-hypoventilation syndrome. There is a dose–response relationship between obesity as expressed by BMI and OHS prevalence (Nowbar et al. 2004). There are data showing that 70–90 % of patients with OHS also exhibit OSAS (Resta et al. 2000). It is in accordance to our study, where 62 % of patients had OHS and 79 % had OSAS.

Most obese patient with respiratory failure can be treated effectively with oxygen or CPAP therapy. But for some seriously ill patients this approach is insufficient. Our trial demonstrated that in such patients NIMV has high efficacy in a

Table 25.2 Baseline spirometry, blood gas analysis and polysomnography in patients with overlap syndrome (OS) and obesity hypoventilation syndrome (OHS)

	OS	OHS	p
VC (ml)	2,487 ± 64	2,290 ±868	NS
VC (%)	68 ± 13	64 ± 17	NS
FEV_1 (ml)	1,680 ± 200	1,870 ± 630	NS
FEV_1 (%)	49 ± 6	61 ± 14	NS
FEV_1/VC %	65 ± 8	78 ± 7	<0.05
SaO_2 (%)	80.3 ± 4.9	75.8 ± 11.1	NS
PaO_2 (kPa)	6.49 ± 0.60	6.04 ± 1.01	NS
$PaCO_2$ (kPa)	9.28 ± 2.41	8.02 ± 1.47	NS
pH	7.34	7.37	NS
AHI	45.0 ± 21.1	47.9 ± 28.1	NS
DI	67.2 ± 25.6	64.3 ± 24.8	NS
Mean minimal nocturnal SaO_2 (%)	71.0 ± 5.0	66.3 ± 10.8	NS

Values are means ± SD
OS overlap syndrome, *OHS* obesity hypoventilation syndrome, *AHI* apnea/hypopnea index, *DI* desaturation index, *FVC* forced vital capacity, FEV_1 forced expiratory volume in 1 s, *NS* non-significant

Table 25.3 Daytime blood gasometry results at baseline, on the fifth day of NIMV, and at 12 months' follow-up NIMV

	Baseline	5th day	12 months
PaO_2 (kPa)	6.19 ± 1.35	8.49 ± 1.79*	8.12 ± 1.21*
$PaCO_2$ (kPa)	8.44 ± 1.88	7.48 ± 1.28*	6.08 ± 0.95*
SaO_2 (%)	76.8 ± 10.1	91.8 ± 3.3*	90.6 ± 3.4*
pH	7.36 ± 0.05	7.38 ± 0.04	7.41 ± 0.04

Values are means ± SD
*$p < 0.05$ compared with baseline

Table 25.4 Clinical variables at baseline and at 12-month follow-up visit

	Baseline	12-month follow-up
BMI (kg/m^2)	47.3 ± 7.9	44.7 ± 6.6
FVC %pred	65.1 ± 15.5	71.9 ± 21.4
FEV_1 %pred	58.3 ± 13.8	64.2 ± 20.8
FEV_1/FVC%	74.5 ± 10.1	70.4 ± 11.0

Values are means ± SD
FVC forced vital capacity, FEV_1 forced expiratory volume in 1 s

short and long period of time. In severe obese patients with chronic and complete respiratory failure, NIMV improved the ventilation during sleep by preventing apneas and hypopneas and increasing the minute ventilation. The control polysomnography performed on the fifth day of NIMV treatment showed significantly higher mean nocturnal oxygen saturation. Interestingly, the daytime gas exchange improved as well, with a significant increase of oxygen saturation and a reduction of carbon dioxide level. Our study demonstrates that after 1 year's nocturnal NIMV performed at home these positive changes were still present and the reduction of carbon dioxide level was even more noticeable. Long term positive effects of NIMV were independent from spirometry changes and loosing weight. Our results confirm the findings of previous small studies of patients who were not as obese, which demonstrated that NIMV causes improvements in daytime gas exchange, daytime somnolence and HRQL (Murphy et al. 2012). Another trial showed that bi-level pressure ventilation improves nocturnal ventilatory control and daytime gas exchange, quality of life, daytime symptoms and daytime physical activity in OHS patients (de Lucas-Ramos et al. 2004); the improvements being associated with subsequent weight loss. However, in contrast to previous data, our study showed that nocturnal treatment

of chronic respiratory failure in patients with severe obesity was not associated with weight loss. We observed some weight reduction, but the change did not reach statistical significance, due likely to a small number of individuals.

During NIMV we observed an increase in the average SaO_2 during sleep, greater in patients with obesity-hypoventilation syndrome than with overlap syndrome (92.5 ± 1.3 *vs.* 90.5 ± 1.9 %, $p < 0.05$). There was a significant improvement of the daytime blood gasometry (Table 25.3). There are several trials that included OHS and COPD patients that showed similar results of NIMV (Murphy et al. 2012; Oscroft et al. 2010). But in some trials nocturnal hypoventilation was better controlled in patient with OHS (Windish and Storre 2012; Storre et al. 2006). Our study also shows that coexistance of COPD in patient with respiratory failure was a prognostic factor of worse efficacy of NIMV.

The duration of the nocturnal NIMV adherence is very important for the treatment efficacy. In our group the mean daily time of NIMV was longer than 5 h. It is in accordance to publication of Murphy et al. (2012) which showed that $\geq$4 h nocturnal ventilation is required to achieve a reduction in daytime carbon dioxide. A careful adjustment of ventilator settings during the nocturnal monitoring is essential for proper control of nocturnal hypoventilation. In our study, NIMV settings were adjusted under the polysomnographic control performed by an experienced technician and a patient was discharged after the optimal treatment had been established. Another reason of high efficacy of NIMV in our study could be more intensive mode of the NIMV treatment. The average percentage of patient-triggered breaths was 45 %, indicating that patients were treated mostly by the controlled mode of NIMV. This is in an agreement with studies of Murphy et al. (2012) and Dreher et al. (2010a, b) showing that the intensive mode of ventilation is more effective in both OHS and COPD patients.

In 28 out of the 34 patients we performed NIMV with ventilator in a pressure preset ventilation mode with the addition of average volume assured pressure support (AVAPS) mode. Treatment tolerance was very high in our study, with only one patient who stopped NIMV because of intolerance in 1 year's observation period. Storre et al. (2006) showed that NIMV conducted with AVAPS mode results in a better control of nocturnal ventilation compared with the pure pressure-preset NIMV in OHS patients. But there are also another data showing no superiority of the AVAPS option over standard pressure-preset NIMV devices (Murphy et al. 2012).

5 Conclusions

OHS and OS belong to the two most common reasons of respiratory failure in severely obese patients. In such a group of patients, NIMV is an effective and well tolerated treatment option resulting in a rapid relief of respiratory disorders during sleep and gradual improvement of gas exchange during the day especially in patients with OHS. The long-term positive effects of NIMV are independent from spirometry changes and loosing weight.

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

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Near-Infrared Hemoencephalography for Monitoring Blood Oxygenation in Prefrontal Cortical Areas in Diagnosis and Therapy of Developmental Dyslexia

26

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Abstract

The purpose of this study was to check empirically the relevance of the near-infrared hemoencephalography (NIR-HEG), which assesses local brain blood oxygenation, in facilitation of the diagnosis and behavioral therapy in dyslexics. The study was carried out in children and teenagers with physiologically recognized dyslexia, of three increasing age-groups: 6–7, 9–10, and 19–20 years old. Healthy age- and gender-matched subjects were used as controls. Left and right prefrontal cortical areas were targeted for the NIR-HEG measurements that were taken at baseline in both controls and dyslexics and then after a 10-day course of midriff breathing exercise combined with a standard vocal and writing training in dyslexics. The major finding was that in dyslexics, irrespective of age, the NIR-HEG indices were lower at baseline compared with those in healthy subjects. We further found that the indices improved after the respiratory and behavioral training in the youngest children, but not in the older age-groups. In conclusion, the study shows that deficient blood oxygenation in the prefrontal cortex is germane to shaping dyslexic symptoms in children. Cortical oxygenation improves in response to respiratory and behavioral therapy in a subset of young dyslexics. The NIR-HEG may facilitate the diagnosis of dyslexic disorder and the monitoring of behavioral therapy, particularly at early age.

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_26,

Keywords
Blood oxygenation • Brain • Breathing exercise • Dyslexia • Infrared hemoencephalography • Prefrontal cortex

1 Introduction

Developmental dyslexia is an increasingly common disorder, although it raises controversies depending on the perspective it is viewed from (Jodzio et al. 2002; Sheets-Johnstone 2003). Dyslexia raises a particular interest when it is viewed from the position of a neurobiologist who tries to introduce newer techniques to study the disorder, doing away with the pedagogical paradigm and focusing instead on brain function and structure considered as a neurofeedback related to cerebral blood flow and, consequently, metabolic activity (Hoshi et al. 1994; Rasey et al. 1996; LeDoux 2000). The relationship between difficulties in acquiring and using language and processes taking place in brain cells is still an area of limited understanding. Hence, gathered empirical material is diverse, making both diagnosis and effective therapy of dyslexic disorders difficult and imperfect.

In the present study we set out to examine whether the non-invasive near-infrared hemoencephalography (NIR-HEG), a technique that assesses local brain blood oxygenation and thus enables an indirect assessment of tissue metabolic activity (Germon et al. 1994; Toomim 2000; Toomim et al. 2004), could be of help in the diagnosis and therapy of dyslexics. Prefrontal cortex is a brain structure that plays an essential role in the regulation of intentional behavior, among others, in cognitive tasks, recognition of novelty grade, emotional feelings and activities, or in the accomplishment of delayed tasks (Raine 1997; LeDoux 2000), but it has not yet been specifically targeted as an area of active role in dyslexia. In the present study we used the NIR-HEG to measure the blood oxygenation level in the left and right prefrontal cortex in three age-groups of dyslexic children and teenagers, the age range 6–20 years, before and after a course of breathing exercise combined with a standard behavioral therapy. We chose the relatively wide age range on the premise that metabolic cortical activity changes with maturation, being likely greater at a younger age of the developmental processes. Overall, we found that cortical blood oxygenation is lower in the dyslexic than healthy subjects, irrespective of age, and it improves in the course of behavioral therapy. The improvement, however, was noticeable only in children younger than 10 years. Thus, NIR-HEG is a non-invasive method that may facilitate the diagnosis of dyslexia. The method may also help monitor therapy in dyslexic children of early age, but fails to have a meaningful bearing at older age in this respect.

2 Methods

The study was approved by an institutional Board for Human Research, performed in accord with the Declaration of Helsinki of the World Medical Association, and informed consent was obtained from the studied children and their parents after explaining the noninvasiveness of the experimental paradigm, but not the research objective of the study. Three groups of children and teenagers, with difficulties in reading, spelling, and writing, consisting of 16 individuals each (8 girls and 8 boys) were studied. The groups were stratified according to age: 6–7 years (Group A), 9–10 years (Group B), and 19–20 years old (Group C). The age- and gender-matched control groups were designated as Group A1, B1, and C1. All measurements were performed in triplicate in each subject, with the mean data of the three taken for statistical elaboration. The results reported herein concern the left and right prefrontal cortical areas. After having acquired the control NIR-HEG measurements, all dyslexic groups underwent a therapeutic training procedure consisting of ten

daily courses of midriff breathing exercise along with the instruction of a 20-min silent and loud syllabic reading, a standard behavioral therapy in dyslexia. The NIF-HEG examination was repeated after the training in like manner. The control subjects had the baseline measurements that were taken as a reference for comparison with the respective groups of dyslexics.

The NIR-HEG opto-technique used is a non-invasive measurement of local tissue oxygenation level, which corresponds to hemodynamic flow changes and thus also follows the changes in metabolic activity of tissue. The technique takes advantage of changes in translucence of the flowing blood due to changes in oxygenation, i.e., hemoglobin – oxyhemoglobin shift, Hb/HbO_2, which generates an electrical signal (Jobsis 1977; Toomim 2000). The uptake of tissue oxygen changes in proportion to metabolic demand, which requires adjustments in the blood oxygen carrying capacity and blood flow. Local changes in oxygenation level measured by NIR-HEG correspond, therefore, to metabolic activity. The measurement gear in this study consisted of a headband spectrophotometer device equipped with two diodes, emitting alternating red (660 nm) and infra-red (850 nm) light through the forehead, corresponding to the active probe and stable basal reference levels, respectively. The scatter of the red light is increased by the HbO_2 formed, while that of the near-infrared light is grossly unaffected. The difference in the amount of the two wavelengths provided by the probes reflects the level of blood oxygenation, which is processed into a signal displayed on a screen. The subjects of the study were able to follow visually oxygenation changes, a positive motivational aspect concerning the persistence in behavioral therapy.

We used an NIR-HEG setup combined with the multimedia biofeedback software FlexComp Infinity/BioGraph Infinity V4.0 by Thought Technology Ltd. (Montreal, Quebec, Canada). Graphical visual signals were processed to yield the following variables:

- *HEG Red* – processed *Red* (crude signal measured in Volts) by means of linear transformation of −0.85/+0.85 V signal into the 0–2 scale, specified as input of red light diode at a wavelength of 660 nm. The value of *Red* depends on the level of HbO_2; the more oxygen saturation, the more red light is reflected;
- *HEG Infrared* – input signal of the diode emitting infrared light at a wavelength of 850 nm;
- *HEG Ratio* – index of regional cortical blood oxygenation, which corresponds to dynamic changes in brain tissue saturation with oxygen;
- *HEG Ratio 5 s* – average/smooth of a 5-s reading of oxygen blood saturation;
- *HEG Ratio Mean* – average index of *HEG Ratio* in the whole session;
- *HEG Ratio Max* – maximum index value *HEG Ratio* during examination;
- *HEG Ratio Min* – minimum index value *HEG Ratio* recorded during examination;
- *HEG Ratio Damper* – average/smooth of a 50-s oxygen blood saturation value, which takes into account hemodynamic changes caused by variations in blood flow speed due to systolic and diastolic pulsation of prefrontal structures; the rhythm correlated with heart rate.

Tabular data are presented as average values. Two-tailed paired or unpaired *t*-tests were used for intra- and inter-group comparisons, as required. $P < 0.05$ was taken as indicative of statistical significant differences.

3 Results and Discussion

Tables 26.1 and 26.2 demonstrate the averaged NIR-HEG data collated from the left and right prefrontal cortical areas, respectively, in the subjects representing all three age-groups studied. There were no consistent differences in the level of oxygenation among the control groups of healthy subjects, although oxygenation tended to be higher in the left prefrontal cortex in the youngest children compared with the older age-groups (Table 26.1); the feature not confirmed in the right hemisphere. Likewise, there were no appreciable differences in the cortical oxygenation among dyslexics before respiratory and behavioral

Table 26.1 Average results of blood oxygen saturation level in the left prefrontal cortex in the three dyslexia age-groups before and after a training course and in their corresponding controls

	A 6–7 years old		A1	B 9–10 years old		B1	C 19–20 years old		C1
	Dyslexia/training		Control	Dyslexia/training		Control	Dyslexia/training		Control
Group	Before	After		Before	After		Before	After	
HEG red	0.44	0.49	0.72	0.40	0.59*	0.61	0.43	0.39	0.59
HEG infrared	1.87	1.87	1.75	1.91	1.87	1.71	1.64	1.48	1.86
HEG ratio	47.6	69.6*	82.4	41.6	42.2	71.9	43.0	45.6	68.8
HEG ratio 5 s	47.5	71.6*	82.3	41.5	41.9	72.0	42.4	47.2	58.7
HEG ratio mean	47.1	55.9*	81.9	41.3	41.7	74.3	49.0	44.8	88.9
HEG ratio max	48.2	73.9*	84.5	42.7	43.7	91.3	50.4	67.6*	99.4
HEG ratio min	45.8	45.1	80.2	39.7	40.9	69.6	47.2	43.1	78.6
HEG damper	47.4	55.4*	82.3	41.6	41.8	73.9	48.9	46.5	77.5

A, B, and C – dyslexics; A1, B1, and C1 – corresponding age-matched controls. All control groups different from the corresponding dyslexic groups before training ($p < 0.01$), except for HEG infrared
*$p < 0.05$ for the training effect in a given age-group of dyslexics

Table 26.2 Average results of blood oxygen saturation level in the right prefrontal cortex in the three dyslexia age-groups before and after a training course and in their corresponding controls

	A 6–7 years old		A1	B 9–10 years old		B1	C 19–20 years old		C1
	Dyslexia/training		Control	Dyslexia/training		Control	Dyslexia/training		Control
Group	Before	After		Before	After		Before	After	
HEG red	0.42	0.45	0.75	0.42	0.40	0.71	0.43	0.45	0.74
HEG infrared	1.57	1.79	1.73	1.80	1.84	1.63	1.82	1.85	1.61
HEG ratio	45.1	63.5*	86.3	44.7	43.5	87.4	45.4	49.2	91.2
HEG ratio 5 s	45.1	68.7*	86.2	44.7	43.8	89.3	45.5	49.7	92.0
HEG ratio mean	44.9	47.3	86.9	45.3	42.7	87.4	46.1	48.7	108.9
HEG ratio max	45.5	88.9*	88.3	50.1	44.5	89.5	63.4	50.3	168.2
HEG ratio min	44.3	42.3	75.2	42.9	41.6	81.7	44.0	47.4	55.4
HEG damper	44.4	47.2	86.1	44.8	43.5	82.6	45.1	49.5	94.4

A, B, and C – dyslexics; A1, B1, and C1 – corresponding age-matched controls. All control groups different from the corresponding dyslexic groups before training, except for HEG Infrared
*$p < 0.05$ for the training effect in a given age-group of dyslexics

therapy, irrespective of age. The dyslexics, however, presented appreciably lower oxygenation indices in both hemispheres, with the exception of the grossly similar reference HEG Infrared measurement, across all age-groups. Cortical oxygenation was nearly halved in the majority of dyslexics before therapy compared with the healthy controls. The corollary is that language processing is one function that involves tasks carried out in the prefrontal cortex. Dyslexia, typically featured as hindered language processing, with ensuing difficulties to master reading, is associated with decreased blood oxygenation in the prefrontal cortex, a sign of calmer metabolic activity and smaller requirement for the oxygen nutrient, and thus also less adaptive hemodynamic adjustments in the area.

The results further show that the therapeutic training consisting of breathing exercises significantly improved the cortical NIF-HEG indices in both hemispheres in the group of the youngest 6–7 years old children Tables 26.1 and 26.2. The increased HEG ratios speak in favor of HbO_2 formation in response to therapy, which translates into better regional tissue oxygenation. However, we failed to show an improvement in the two older age-groups. The lack of a measurable improvement in oxygenation in the latter age-groups is not readily explainable. A viable explanation may be that younger children have a

greater ability to use brain plasticity to adjust function, which would also entail a greater brain metabolic activity and, consequently, vascularization and blood flow in response to training. The training therapy was kept meticulously the same in all age-groups studied. There might be, however, differences in motivational aspects concerning the upholding of the interest in therapy to the disadvantage of the older subjects, which were not controlled for and thus might have gone unnoticed.

Prefrontal cortex has been incriminated in a number of pschyophysiological functions. Raine (1997), searching for factors triggering aggression, has pointed to an impairment of blood flow in the prefrontal region as a probable cause of lack of self-criticism, emotional swings, lack or excess of fear directed at self, all of which characteristic of psychopathic personalities. Other studies have linked the frontal lobes to the optimal integration and control of executive functions (Toomim and Toomim 1999; LeDoux 2000; Dalgleish 2004). These cortical structures appear germane to the monitoring of behavior and its adjustments, control over the performance of goal-oriented tasks, and psychological flexibility that helps solve the arising issues (Havas et al. 2007; Spielberg et al. 2008). The present study adds to this roster of functions played by prefrontal cortex its plausible role enabling a proficient language and related skills processing. Our study could not discern whether decreased metabolic activity in the prefrontal regions is the underlying cause triggering dyslexic disorders or is a secondary reflection of dyslexia-blunted brain activity emanating from other brain structures. Nevertheless, the results point to an attractive possibility of a relatively easy and cost-effective method of measuring the prefrontal cortex oxygenation, as a confirmatory measure of the true existence of dyslexic disorders, particularly in dubious cases.

4 Summary

The study lends support to the notion that deficient performance of prefrontal cortex is germane to shaping dyslexic disorders in children and teenagers. The finding that dyslexia may have to do with insufficient cortical oxygenation contributes to a better understanding of the pathomechanism of developmental dyslexia and opens up a new avenue of training therapy directed at the enhancement of oxygen delivery, such as breathing exercise, and thus the improvement of metabolic brain tissue activity. The infra-red hemoencephalography appears a useful tool that may help confirm the presence of dyslexia in doubtful cases and monitor the effectiveness of therapy. An attractive possibility arises that cortical oxygenation level, assessed non-invasively through the scalp, may be a marker of dyslexia; an idea that warrants exploration in further research.

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

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Occlusal Stabilization Splint Therapy in Orofacial Pain and Tension-Type Headache

27

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Abstract

Studies suggest an association between orofacial pain, accompanying temporomandibular disorders of myogenous origin, and headache, especially its tension-type. The occlusal appliance therapy is one of the options for the treatment of orofacial pain due to masticatory muscles tenderness. The aim of the present study was to assess the effectiveness of occlusal stabilization splint therapy in myofascial pain and tension-type headache in patients with sleep-disordered breathing. Forty three such patients were enrolled into the study group. The patients were treated with stabilization occlusal splint of vertical thickness at vertical jaw separation, established individually for each patient using a cephalometric analysis. The intensity of orofacial pain (numeric rating scale) and headache (analog rating scale), frequency of headache (%), and jaw qualitative function were assessed at baseline and after 2 and 6 months. Medians of headache and orofacial pain intensity were reduced after 6 months of treatment compared with baseline: 6.0 vs. 2.0 ($p < 0.0001$) and 6.0 vs. 1.0 ($p < 0.0001$), respectively. Pain decreased below 3 score points in 61.8 % of the patients with headache ($p = 0.23$) and in 85.3 % of patients with orofacial pain ($p < 0.0001$). Overall, the improvement in both signs and symptoms of orofacial pain was observed 81.4 % of patients after using occlusal stabilization splint for 6 months. We

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_27,

conclude that occlusal stabilization splint was effective in reducing painful symptoms of temporomandibular disorders of myogenous origin, a frequent feature of sleep disordered breathing.

Keywords
Occlusal stabilization splint • Orofacial pain • Temporomandibular disorders • Tension-type headache

1 Introduction

Temporomandibular disorder (TMD) is a term embracing clinical conditions that involve disturbances in the masticatory muscles, temporomandibular joints, and the associated structures (Dworkin and Le Resche 1992). These disorders are characterized by pain in the preauricular area, temporomandibular, or masticatory and craniofacial muscles, limitation, or deviation in the mandibular movement, and temporomandibular sounds during mandibular function. Head and neck pains are also common complaints. Patients frequently suffer primary headaches, such as migraine or tension-type headache. It is suggested that tension-type headache is closely associated with the prevalence of myofascial pain, the masticatory muscles origin type of TMD (Jensen and Olesen 1996). The etiology of TMD is multifactorial, and the latest theory focuses on the 'biopsychosocial' approach, which means the combined influence of biological, psychological, and social problems (Greene 2001). The main responsibility of myofascial pain signs and symptoms bears the disturbances within the masticatory muscles of different origin (direct or indirect trauma, strain injury, localized tissue injury) (Cairns 2010). The major signs and symptoms of myofascial pain are diffuse orofacial pain, masticatory muscle tenderness, impaired mandibular movement, and headache, mainly morning headache in the temporal region. The treatment approach comprises behavioral therapy, psychopharmacological, and physiotherapy. The use of occlusal stabilization splint is recommended for muscle relaxation. It has been shown that splints decrease the masticatory muscles electromyographic activity and increase its symmetry (Ferrario et al. 2002). This could be explained by the effects of the occlusal support consisting of increasing the length of muscle fibers and unloading temporomandibular structures. The effectiveness of occlusal stabilization splint therapy in reducing signs and symptoms of myofascial pain, mainly in decreasing masticatory muscles pain, has been reported by many authors (Turp et al. 2004). However, the mechanisms of that effect are unclear. These mechanisms do not necessarily have to do with counteracting bruxism by occlusal splints, as bruxism does not exclusively underlie masticatory muscle pain (Svensson et al. 2008; Holmgren et al. 1993).

Tension-type headache also represents pain of primary origin (Headache Classification 2004), and stress and psychological disturbances are its main etiological factors. The relationship between myofascial pain and tension-type headache has not been fully elucidated, but a sustained tooth clenching is suggested to be a potential aggravating factor for both (Jensen and Olesen 1996), which is explained by increased sensitivity to afferent stimuli in patients with headache. The treatment approach in the diagnosed tension-type headache is similar to that applied in myofascial pain, and it involves behavioral and stress management, and the use of analgesics (Dahlof and Jakobs 1996). Headache lasting for more than 3 months and occurring with increasing frequency leads to a chronic type of headache. It seems advisable to develop a tool for decreasing both the intensity and frequency of this type of headache.

The aim of the present study was to assess the effectiveness of occlusal stabilization splint

therapy in orofacial pain in patients with myofascial pain and tension-type headache during a 2- and 6-month follow-up.

2 Methods

2.1 Subjects

The study was approved by the Bioethical Committee of the Medical University in Warsaw, Poland, and informed consent was obtained from study participants. Forty-three patients (38 women and 5 men), aged 18–59 (mean age of 32.7 ±9.4 years), were included in the study based on the chosen criteria of the myofascial pain and tension-type headache. The study group was recruited from the patients referred to the Department of Prosthodontics of Warsaw Medical University for the treatment of TMD symptoms. The exclusion criteria were as follows: temporomandibular joint pain, symptoms related to other diseases of the stomatognathic system, non-specific symptoms, psychiatric, psychological, and neurological disorders, sleep-disordered breathing, regular drug and alcohol intake, history of trauma or tumor, and unstable dental condition.

Myofascial pain was diagnosed according to the research diagnostic criteria for temporomandibular disorders (RDC/TMD) of Dworkkin and Le Resche (1992). Orofacial pain of muscle origin was recorded, including pain associated with tenderness to palpation of localized areas of the masticatory muscles. On the basis of clinical examination, myofascial pain was recognized without (Group Ia) and with limited opening (Group Ib). Tension-type headache was diagnosed on the basis of history questionnaires and clinical examination performed in accord with the diagnostics criteria for it (Headache Classification 2004). The headache must have been present for more than 1 day but less than 15 days per month for the last 3 months, with bilateral location and with pericranial tenderness (Jensen and Rasmussen 1996). No neurological evaluation was performed. The information about the general health, complaints' duration and frequency, parafunctions, and face and head pains was taken from the medical history data.

2.2 Study Design

This study was designed as a longitudinal follow-up. History questionnaires were completed and clinical examination was performed according to RDC/TMD before treatment and after 2 and 6 months of follow-up. In all patients, a lateral cephalogram was obtained to determine cephalometric variables related to the craniofacial morphology and vertical jaw separation for vertical thickness of occlusal stabilization splint.

The intensity of headache and orofacial pain was recorded using numerical rating scale ranging from 0 (no pain) to 10 (pain as bad as could be). The characteristics of orofacial pain before and 6 months after treatment were based on history questionnaires and Axis II RDC/TMD scoring protocol for the characteristic pain intensity. Treatment effectiveness was assessed subjectively and objectively at check-up visits. The patients used the NRS to evaluate their pain intensity and jaw functioning questionnaire to assess the overall improvement and define their feelings as one of the following: symptom free, much better, better, unchanged, and worse. The jaw functioning examination allowed for the clinical assessment of the masticatory organ function as follows: no signs of myofascial pain, any pain but impaired mandibular movement, and temporomandibular joints sporadic sound during mandibular movement unchanged or worse.

2.3 Occlusal Splint

Each patient had the hard acrylic occlusal stabilization splint prepared for night use. The splints were for the lower jaw and covered all of the teeth. The splint surface for contact with opposite teeth was flat. The maxillary teeth contacts with the splint were adjusted as in 'ideal' occlusion in centric relation: multipoint contacts with

posterior teeth and anterior guidance. The centric relation was achieved by the chin-point guidance. The vertical thickness of the splint was established in vertical jaw separation (VJS) determined in the cephalometric analysis. Individual VJS determination for vertical thickness of the splint was examined in our previous study (Kostrzewa-Janicka et al. 2012). It was revealed that the treatment was most affective after using the splint of vertical thickness at VJS of the patient's minimum bite force. The bite force was measured bilaterally at different VJS. The course of bite force at different VJS was analyzed depending on the skeletal morphology based on cephalometry. The regression analyses allowed for determining the formula of VJS in minimal bite force for each patient under cephalometry as follows:

$$\begin{aligned} \mathrm{VJS} = {} & -115 + 0.089 \cdot \mathrm{a}(\mathrm{SE} = 0.035) + 2.76 \\ & \cdot \mathrm{b}\ (\mathrm{SE} = 1.16) - 0.016 \\ & \cdot (\mathrm{b})^2(\mathrm{SE} = 0.007) - 0.002 \\ & \cdot (\mathrm{c})^2(\mathrm{SE} = 0.0007) \end{aligned}$$

where:

a = Ar-Go-Me; b = vPUK-Go; c = NSL-ML (articulare–gonion–menton as a gonion angle;

Go-vPUK, as the length of mandibular body;

vPUK as the point placed on ML at the end of perpendicular line from pogonion;

NSL-ML as the inclination of the mandible in relation to the anterior cranial base;

where:

S is sella point, Go is gonion point, N-nasion, Ar–articulare, Me–menton, and ML–mandible base).

To establish the proper vertical mandibular position for the vertical thickness of occlusal stabilization splint, the value of VJS from the equation was added to the measured distance between the incisal edge of first upper incisal and gingival edge of first lower incisal in maximal intercuspal position. In the articulator Reference Sl (Gamma Dental, Austria), the lower occlusal stabilization splint was made. The splint had a flat occlusal surface that was in contact with all supporting teeth and anterior guidance. The mandibular position was in centric relation achieved by chin-point guidance.

The primary end-points in the study were to assess the intensity of headache, orofacial pain, and CPI at baseline and after 2- and 6-month of follow-up. The secondary end-points were to achieve a sufficient predetermined level of treatment effectiveness of no more than 20 points in the characteristic pain intensity scale and less than 3 points in the numerical rating scale for orofacial pain and headache.

2.4 Statistical Analysis

Statistical analysis was performed by means of SAS/STAT® 12.1. (2012) and van Belle's et al. (2004) handbook. All end-points were presented as medians with lower and upper quartiles or as a percentage of events below predetermined thresholds. A signed rank test was used to compare second and sixth month of follow-up with respect to the levels of the end-points considered. A binomial proportion with 95 % CI was determined for the percentage of events of end-points to compare 1–50 %. Spearman's correlation coefficients were determined to reflect associations between the measurements of intensity of end-points.

3 Results

The study group was mostly composed of patients with normal occlusion in the complete natural dentition (Table 27.1). Before the treatment, the medians of intensity of headache, orofacial pain and characteristic pain intensity were 6.0, 6.0, and 60, respectively, with 1–8 episodes of headache per month. Check-up Visit I after 2 months was attended by 40 patients and Visit II by 34 patients after 6 months.

After 6 months of night-time use of the lower occlusal stabilization splint, there were sixfold, threefold, and sixfold decreases, compared with

Table 27.1 Baseline characteristics of patients

Myofascial pain:	
Ia	32 (74.4 %)
Ib	11 (25.6 %)
Angle's classification of malocclusion:	
1	35 (81.4 %)
2	4 (9.3 %)
3	3 (7 %)
Open bite	1 (2.3 %)
Eichner's classification:	
Group A	42 (97.7 %)
Group B	1 (2.3 %)
Group C	0 (0.0 %)
General disorders:	
Yes	16 (37.2 %)
No	27 (62.8 %)
Skeletal disorders:	
Yes	10 (23.3 %)
No	33 (76.7 %)
Characteristic pain intensity (score)	60 (47–66)
Intensity of orofacial pain (numeric rating scale)	6 (4–7)
Intensity of headache (numeric rating scale)	6 (5–7.9)
Frequency of headache:	
Once a month	7 (16.3 %)
Two to four times per month	11 (25.6 %)
At least five times per month	25 (58.1 %)

Data are given as number (%) or median (Q1–Q3). General disorders reported by patients: hormonal disorders (sex hormones, thyroid hormones), skeletal disorders, cardio-vascular disorders, digestive system disease, rheumatoid disease, and boreliosis

Table 27.2 Levels of end-points during 6-months' follow-up (median Q1–Q3)

	Baseline	After 2 months	After 6 months	Statistical comparisons		
	0	2	6	2 vs. 0	6 vs. 0	6 vs. 2
CPI	60.0 (47.0–66.0)	–	10.0 (0–23.0)	–	0.001	–
OFP	6.0 (4.0–7.0)	1.3 (0–2.7)	1.0 (0–2.2)	0.001	0.001	0.036
HA	6.0 (5.0–7.9)	3.0 (0–4.0)	2.0 (0–3.0)	0.001	0.001	0.053
Frequency of HA	3.0 (2.0–3.0)	1.0 (0–1.0)	1.0 (0–1.0)	0.001	0.001	0.750

CPI characteristic pain intensity, *OFP* orofacial pain, *HA* headache

baseline, regarding the intensity of characteristic pain intensity, headache, and orofacial pain, respectively. Significant decreases in the intensity of end-points were already found after 2 months of treatment. Despite a sustained downward tendency, the difference in the score of the numbering rating scale for headache and orofacial pain between check-up Visits I and II was not statistically significant. There was a threefold decrease of headache frequency at the last check-up visit compared with baseline (Table 27.2).

An improvement in both signs and symptoms of myofascial pain, as assessed by the jaw functioning examination, was observed in 81.4 % of patients after 6 months of splint usage. A decrease in the score of the characteristic pain intensity below 20 points was found in 73.5 % of the

Table 27.3 Percentage of patients under the predetermined endpoint level

	After 2 months	After 6 months
Decreasing of CPI under 20 points	–	73.5 % (55.6–87.1)[a]
Decreasing of OFP under three points	77.5 % (61.6–89.2)[a]	85.3 % (71.9–98.7)[a]
Decreasing of HA under three points	45.0 % (29.3–61.5)	61.8 % (43.6–77.8)

Data are given as % (95 % confidence intervals)
CPI characteristic pain intensity, *OFP* orofacial pain, *HA* headache
[a]Significant difference for comparison with 50 %

patients (significantly exceeded 50 %; $p < 0.01$) (Table 27.3). After a 2-month observation, the numeric rating score decreased below 3 points for headache in 45 % of patients ($p = 0.64$) and for orofacial pain in 77.5 % of the patients (significantly exceeded 50 %; $p < 0.0009$). Similar results were observed after a 6-month period for headache and orofacial pain intensity in 61.76 % (significantly exceeded 50 %; $p < 0.01$) and 85.3 % of the patients (significantly exceeded 50 %; $p < 0.0001$), respectively.

The intensity of headache during the 2- and 6-month observation significantly correlated with the frequency of pain ($r = 0.81$, $p < 0.0001$ and $r = 0.87$, $p < 0.0001$, respectively), which was not observed before treatment ($r = 0.24$, $p < 0.12$). Also, a decrease of headache and orofacial pain intensity between baseline and Visit I correlated positively ($r = 0.55$, $p < 0.0001$). An analogous finding was observed between baseline and Visit II ($r = 0.66$, $p < 0.0001$).

4 Discussion

The study demonstrates that the occlusal stabilization splint is an effective device for the management of myofascial pain and tension-type headache. Treatment directed toward amelioration of masticatory muscles disturbance also had a positive effect on headache. This corresponds well with the results of Ekberg and Nilner (2006). Although, the orofacial pain was the main complaint of patients seeking medical care, they also reported episodes of intensive headache (medians of orofacial pain and headache = 6). Admittedly, the use of occlusal splints reduced the median for orofacial pain intensity to the values lower than those of the median for headache intensity, both after 2 and 6 months of observation (medians = 1.3 vs. 3 and 1 vs. 2). However, reductions in headache complaints were comparable with the decreased pain intensity reported after application of some analgesics (Dahlof and Jakobs 1996). Patients suffering from recurrent tension-type headache reported, additionally, a significant reduction in the frequency of headache during the treatment time; the finding also being in line with that of other authors (Ekberg et al. 2002; Ekberg and Nilner 2006).

After 2 months of wearing occlusal stabilization splint, the decrease in headache intensity corresponded in 30 % with that in orofacial pain intensity and *vice versa* (r^2 of 30). A significant and satisfactory alleviation of pain was observed already after a 2-month occlusal stabilization splint therapy. The difference in complaints after 2 and 6 months was statistically insignificant, although its downward tendency was maintained (medians for orofacial pain = 1.3 vs. 1; medians for headache = 3 vs. 2, respectively). These results indicate that a 2-month observation is enough to assess the effectiveness of the use of occlusal splint; the finding that has not been reported in other studies. Other studies provide, generally, results regarding the assumed end-points, without a comparison between the check-up visits (Ekberg and Nilner 2006; Sheikholeslam et al. 1993). The present study demonstrates that if the use of an occlusal stabilization splint does not give a satisfactory improvement over a 2-month observation period, then the approach should be changed to look for the aggravating factors, such as stressful life-events or general disorders.

The duration of occlusal splint therapy influenced the patients' ability to assess the perception of headache. No correlation between the intensity of headache and its prevalence was noted at the first check-up visit contrary to consecutive visits. This could result from a lower prevalence of complaints due to the therapy applied, which, in turn, made easier for the patients to assess the perception of pain during painful episodes.

The influence of general condition and fluctuations in local factors on the treatment effectiveness should be kept in mind, particularly considering that etiological factors for orofacial pain and tension-type headache are similar and could be of central or local origin, with a quite important contribution of stress and parafunctional activities (Svensson and Graven-Nielsen 2001; Jensen and Olesen 1996). Jensen and Olesen (1996) demonstrated that the patients with tension-type headache more frequently develop the headache following sustained tooth clenching than the healthy controls do. A decrease in parafunctional activities by occlusal splint could explain the decrease of headache frequency (Glaros et al. 2007; Klasser et al. 2010). A reduction in rhythmic masticatory muscle activity is a partial explanation of a significant reduction in morning headache and orofacial pain intensity after the use of specially designed splints, the mandibular advancement appliance (MAA) (Franco et al. 2011). The MAA are mainly recommended for patients with sleep disordered breathing (Rodríguez-Lozano et al. 2008; Fransson et al. 2003). After the MAA use, a reduction in both patients' complaints (snoring, apnea, daytime tiredness, and poor quality of sleep) and the value of oxygen desaturation index were observed.

A significant decrease in the intensity and prevalence of tension-type headache observed in the present study after the use of occlusal stabilization splints suggests that such splints are useful as additional therapeutic elements and may also be helpful in the differential diagnosis of headaches.

5 Conclusions

1. Occlusal stabilization splint therapy met expectations in terms of effectiveness in reducing signs and symptoms in patients with myofascial pain disorder.
2. It seems essential to apply occlusal stabilization splint therapy even in those patients who report moderate intensity of myofascial pain with concomitant tension-type headache.
3. Occlusal stabilization splints can be an additional useful tool in the diagnosis of tension-type headache.
4. Occlusal stabilization splint could also be used as a safe alternative method for managing the tension type headache in the general population.

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

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Dynamics of Upper Airways During the Müller Maneuver in Healthy Subjects: A Cine MRI Study

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Isato Fukushi and Yasumasa Okada

Abstract

The Müller maneuver has been widely applied to mimic the pathophysiological condition of obstructive sleep apnea (OSA) during wakefulness. We applied cine MRI to elucidate dynamics of the upper airway during the Müller maneuver in healthy subjects (n = 7). Three sets of images (during quiet nose breathing, quiet mouth breathing, and Müller maneuver) were recorded on sagittal midline plane together with impedance pneumography. The position of the tongue root changed during a respiratory cycle when subjects breathed quietly. At the early inspiratory phase the tongue root moved forward and upward, the retroglossal airway size increased toward the middle of inspiration, and the airway size became smaller again toward the end of inspiration. During expiration the airway size became further smaller. When the subject performed the Müller maneuver, the movement of the oropharynx and its narrowing were greater than those of the velopharynx. However, the airway was not completely obstructed. A relatively large morphological change was observed in the retropalatal and retroglossal regions with the backward and downward motion of the tongue root and flattening of the tongue shape during the Müller maneuver. Although patterns of upper airway narrowing and tongue shape alterations were variable among subjects,

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_28,

upper airway narrowing was commonly prominent in the retroglossal area. Cine MRI with the Müller maneuver enables to visualize the upper airway dynamics and could be easily applied to evaluate upper airway collapsibility during wakefulness.

Keywords
Obstructive sleep apnea • Mouth breathing • Müller maneuver • Nose breathing • Tongue • Upper airway dynamics

1 Introduction

Obstructive sleep apnea (OSA) emerges due to periodic narrowing or occlusion of the upper airway during sleep (Dempsey et al. 2010). To evaluate the structural changes of the upper airway in patients with OSA, static imaging such as X-ray cephalometry, computed tomography scanning, and magnetic resonance imaging (MRI) have been performed (De Backer et al. 2008; Thakkar and Yao 2007). However, the morphology of the upper airway changes dynamically during a single respiratory cycle, so the static imaging does not provide sufficient information on the upper airway dynamics. To dynamically image the upper airway, various attempts have been reported such as fluoroscopic and echographic imaging (Stuck and Maurer 2008) as well as endoscopic observation (Ko and Su 2008; Launois et al. 1993). Endoscopically, however, it is possible to observe only the dynamics of the airway lumen surface, and that of the soft tissue surrounding the upper airway cannot be evaluated. Compared with these techniques, cine MRI has various advantages such as non-invasiveness, and enables to widely visualize the dynamics of the upper airway and its surrounding tissue. Indeed, cine MRI has been successfully applied to analyze the dynamics of upper airways during sleep in patients with OSA (Barrera et al. 2010; Chuang et al. 2009; Moriwaki et al. 2009; Donnelly et al. 2003).

To evaluate the dynamics of the upper airway during sleep, drugs such as propofol are often administered to induce sleep (Rabelo et al. 2010; Chuang et al. 2009). However, drug-induced sleep may be different from natural sleep in its nature (Rabelo et al. 2010), and drugs such as propofol preferentially suppress the genioglossal activity and abnormally increase collapsibility of the upper airway (Eastwood et al. 2005). Drug-induced sleep may also cause pathological respiratory suppression, and should not be applied to hypoventilating patients (Kashiwagi et al. 2004). To avoid the complexity to induce sleep in OSA patients, the Müller maneuver, which consists of a forced inspiratory effort with the mouth and nose closed, could be applied as substitute of real sleep. This maneuver can mimic the pathophysiological condition of OSA during wakefulness, and has been applied to the studies of OSA (Ko and Su 2008; Gregorio et al. 2007; Liao et al. 2003; Terris et al. 2000; Jäger et al. 1998). A successful example of the Müller maneuver application is the evaluation of upper airway function by fiberoptic nasopharyngoscopy with this maneuver before surgical intervention to predict surgical outcome and to improve patient selection (Sher et al. 1985). Although the Müller maneuver has been widely applied to these studies, yet the dynamics of the upper airway during the maneuver has not been fully clarified even in healthy subjects. In the present study, therefore, we applied cine MRI to elucidate the dynamics of the upper airway and its surrounding tissue during the Müller maneuver and during a eupneic respiratory cycle in healthy subjects.

2 Methods

This study was conducted with the approval of the Ethics Committee of Keio University Tsukigase Rehabilitation Center. Seven male healthy subjects voluntarily participated in this study. None had a history of a major respiratory disorder. The ages and the body mass indices of the subjects were 30.6 ± 6.8 (mean ± SD) and 22.1 ± 2.4 kg/m^2, respectively. None had contraindication for MRI. Dynamic images of the upper away and its surrounding tissue were acquired by cine MRI. MRI was performed using a 1.5-Tesla MRI using a QD CTL Spine array coil and CTL Anterior bridge (EXCELART Vantage AGV, Toshiba Medical Systems, Ohtawara, Japan). The sequences were as follows: (1) Sagittal locator on the midline: spin echo (SE), repetition time (TR)/echo time (TE) = 60/10 ms, field of view (FOV) = 27 × 27 mm^2, slice thickness (THK) = 8 mm, number of excitation (NEX) = 1; (2) Coronal locator: spoiled gradient echo (SPGR), TR/TE = 100/10 ms, FOV = 27 × 27 mm^2, THK = 8 mm, NEX = 1; (3) Three sets of images (i.e., during quiet nose breathing, quiet mouth breathing, and the Müller maneuver) were taken on sagittal midline plane; in the velopharynx, glossopharynx, and hypopharynx, with the following technique: field echo (FE) with multiphase, TR/TE = 12.5/5.5 ms, flip angle (FA) = 25°, band width (BW) = 163 Hz, FOV = 25 × 25 mm^2, THK = 12 mm, phases = 120 (for 108 s), matrix = 32 × 96, NEX = 1.

Each subject lay supine with their head in the neutral position. All recordings were conducted with the eyes of the subjects open, so that they were awake. A respiratory band for impedance pneumography was placed around the upper abdomen, and the abdominal wall motion accompanied by breathing was monitored on an oscilloscope for determination of respiratory phase and digitally recorded simultaneously with cine MRI. MRI recording was performed in the order of quiet nose breathing, quiet mouth breathing and Müller maneuver for 108 s each. Regarding the Müller maneuver, subjects were requested to make a maximal effort to inspire with their nose and mouth closed for 6–7 s three times during the 108 s recording course (at 30, 60, and 90 s after the recording started).

Acquired cine MRI data were transferred into DICOM format file and analyzed using a software zioTerm2009 (Ziosoft, Tokyo, Japan). On the midline sagittal images, the coordinates of a point on the tongue root (point A, Fig. 28.1) and another point that is static on the most infero-posterior portion of the third cervical vertebra (point B, Fig. 28.1) were taken and the distance between these two points was calculated to evaluate the tongue movement. The respiratory phase of each

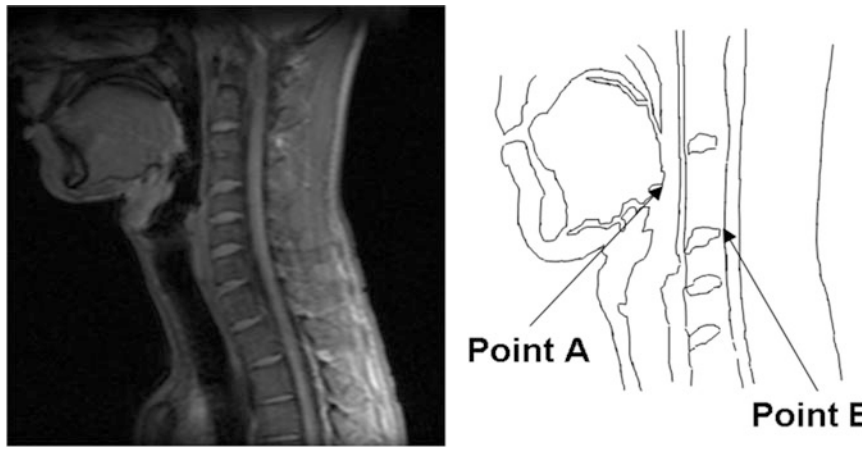

Fig. 28.1 MR image on the midline sagittal plane (*left panel*) **and its schema** (*right panel*). *Point A*, tongue root; *Point B*, the most infero-posterior portion of the third cervical vertebra

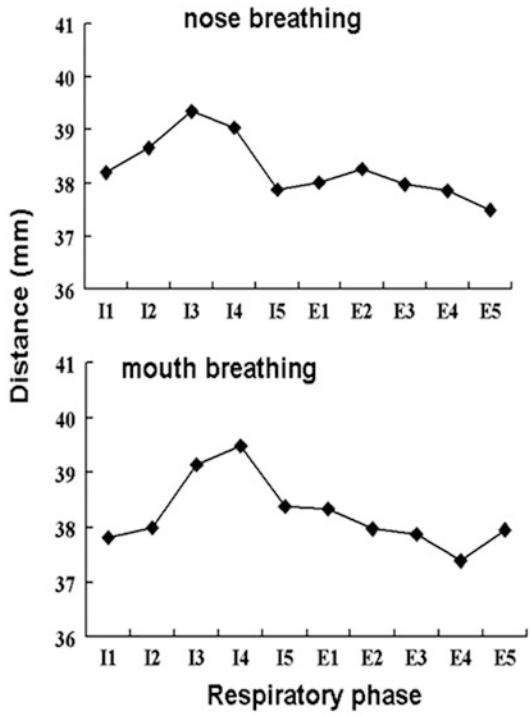

Fig. 28.2 The distance between the tongue root and the third cervical vertebra in each divided respiratory phase. For example, *I1*, the first inspiratory phase; *I3*, the third inspiratory phase; *E5*, the 5th expiratory phase

image was determined based on the simultaneously recorded impedance pneumogram. A single respiratory cycle was divided into inspiration and expiration. Further, each of the inspiratory and expiratory phase was equally divided into five subphases, i.e., into I1, I2,·. . ., I5 and E1, E2,·. . ., E5, respectively. The average distance between points A and B was calculated in each subject at each subphase during a single respiratory cycle.

3 Results

The position of the tongue root changed during each respiratory cycle when the subjects breathed quietly. At the early inspiratory phase, the tongue root gradually moved forward and upward, and the distance between points A and B was gradually increased until the middle of inspiration. Then, the distance became shorter again toward the end of inspiration. During expiration the distance gradually became shorter, and it was shortest at the end of expiration. Cyclic changes of the tongue position synchronized with respiration were observed. Although the changes in the position are small during quiet breathing, the upper airway is most patent at the middle of inspiration. These dynamics were commonly observed during both nose and mouth breathing (Figs. 28.2 and 28.3). However, it was noted that, compared with nose breathing, the tongue was elongated more along the antero-posterior axis during mouth breathing.

Displacement of the tongue and soft tissue around the velopharynx and oropharynx was visible on MR images when the subject performed the Müller maneuver; the movement of the oropharynx and its subsequent narrowing were larger than those of the velopharynx. However, the upper airway was not completely obstructed, and a considerable patency was maintained in all subjects (Fig. 28.4). A relatively large morphological change was observed in the retropalatal and retroglossal regions including the backward and downward motion of the tongue root; due to the backward traction the tongue became flattened during the Müller maneuver. Although patterns of upper airway narrowing and tongue shape changes were variable among subjects, upper airway narrowing was commonly prominent in the retroglossal region in the studied subjects (Fig. 28.4).

4 Discussion

In the present study, the position of the tongue root showed the largest displacement during quiet nose and mouth breathing; the distance between the tongue root and the third cervical vertebra was largest at the middle of inspiration and smallest at the end of expiration. Kalra et al. (2006) conducted cine MRI during normal breathing and reported that the airway volume was largest at the peak of inspiration and smallest at the end of expiration. Our results of airway size dynamics were principally in agreement with Kalra et al.

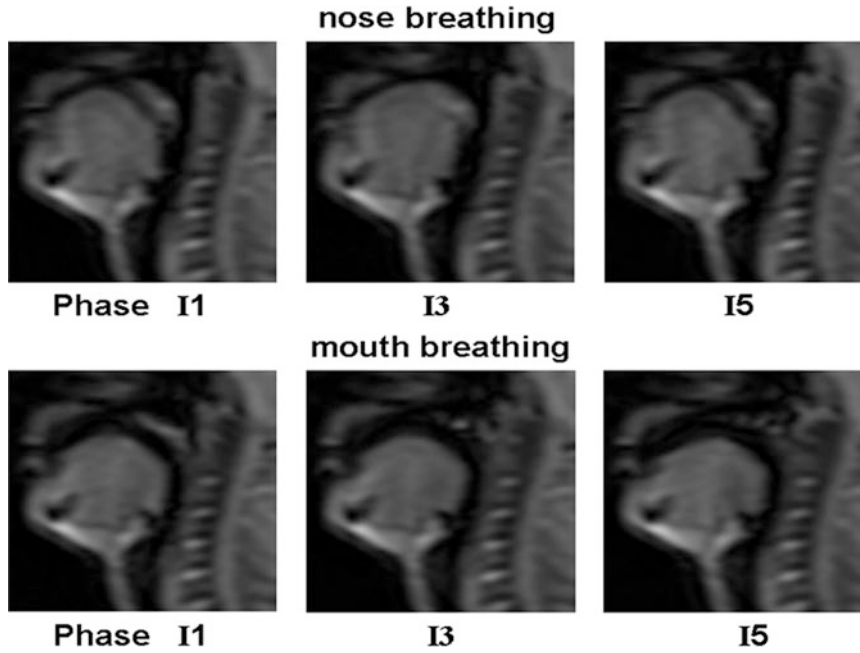

Fig. 28.3 Cyclic changes of the tongue position during a respiratory cycle. *Upper panel*, nose breathing. *Lower panel*, mouth breathing. The tongue positions at the beginning of inspiration (*I1*), middle of inspiration (*I3*) and end of inspiration (*I5*) are shown. Although the changes in the position are small during quiet breathing, the upper airway is most patent at I3. Compared with nose breathing, the tongue is elongated more along the antero-posterior axis during mouth breathing

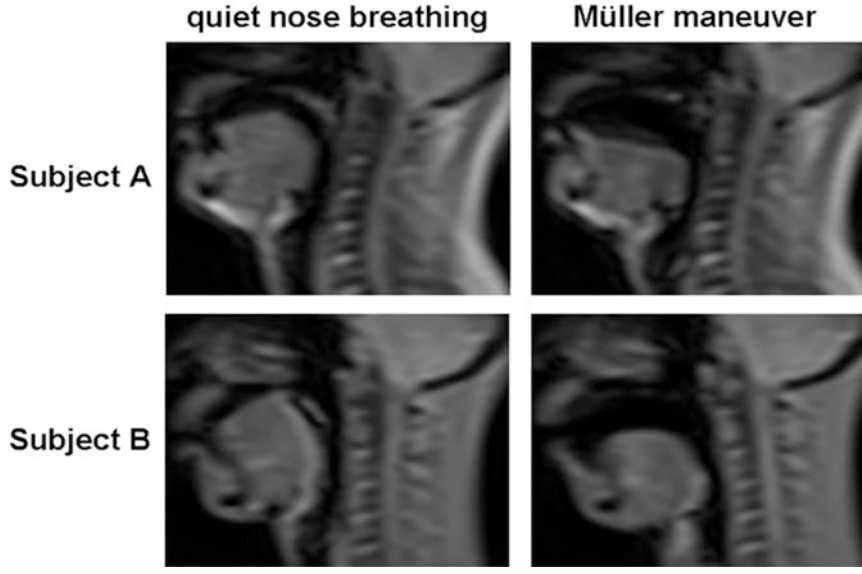

Fig. 28.4 Examples of MR images in two representative subjects before and during the Müller maneuver. In both subjects the tongue moved posteriorly and became flattened, but the patterns of airway narrowing were different. In *Subject A* both the retropalatal and retroglossal regions were narrowed. In *Subject B* only the retroglossal region was narrowed

(2006), although our analysis was two-dimensional and that by Kalra et al. (2006) was three-dimensional. The genioglossus moved anteriorly during inspiration and posteriorly during expiration with various degrees of motion among subjects in our study. Cheng et al. (2008) reported that the genioglossus moved anteriorly during the early phase of inspiration and moved posteriorly again toward the end of inspiration. Our results (Figs. 28.2 and 28.3) coincided with the observation by Cheng et al. (2008). Compared with nose breathing, the motion of the genioglossus, and also that of the tongue root, was greater during mouth breathing, although the patterns of tongue root movement during nose and mouth breathing were principally the same. The movement of the tongue synchronized with respiration would contribute to the optimization of the upper airway patency during a single respiratory cycle (Saboisky et al. 2006). Ciscar et al. (2001) reported that the patent area in the velopharynx is smaller in OSA patients than in control healthy subjects only transiently during a single respiratory cycle and that this difference increases during sleep. Our analyses were conducted in healthy subjects, and it must be noted that different findings might be obtained in patients with OSA. Our observation that the movement of the tongue root is larger during mouth breathing than during nose breathing accounts for the tendency that upper airway obstruction occurs more frequently during mouth than nose breathing (Fitzpatrick et al. 2003). This consideration is in agreement with the previous report that the anatomical states of the oral cavity and pharynx during mouth breathing is more disadvantageous compared with nose breathing to maintain the upper airway patency.

Jäger et al. (1998) observed obstruction of the velopharynx and oropharynx during the Müller maneuver in patients with OSA, but not in healthy subjects, and concluded that healthy subjects do not sustain any severe narrowing along the pharyngeal airway. Our observation with the Müller maneuver is in agreement with that of Jäger et al. (1998). Ritter et al. (1999) found by fiberoptic nasopharyngoscopy with the Müller maneuver that the upper airway patency was progressively reduced in the retropalatal region, as the intraluminal pressure decreased from −10 to −40 cm H_2O. In their observation, the retroglossal area did not change with the Müller maneuver, although the airway configuration altered. White (2005) reported that the genioglossus briskly responds to negative pressure in healthy subjects, which should prevent the collapse of the upper airway.

Each patient with OSA has a distinct pattern of narrowing or collapse of the pharynx (Chuang et al. 2009). Moriwaki et al. (2009) reported that velopharynx was identified as the most frequent site of obstruction among subjects with OSA. In our observation with the Müller maneuver, narrowing in the oropharynx tended to be larger than that in the velopharynx, although the pattern and anatomical portion of the upper airway narrowing with the Müller maneuver were not uniform among subjects in our study. Although the narrowing patterns of the upper airway in patients with OSA during sleep and wakeful states are not completely the same (Chuang et al. 2009), the Müller maneuver could be efficiently and easily applied to evaluate the upper airway obstruction in patients with OSA (Sher et al. 1985). Therefore, with sufficient caution, cine MRI with the Müller maneuver could be used as one of the helpful supportive means to evaluate upper airway collapsibility in OSA patients because the potential obstruction sites could be easily and safely detected in the wakeful state.

Acknowledgement The authors thank Mr. Manabu Inose for his technical assistance and Dr. Kanehiro Fujiyoshi for his critical reading of the manuscript. This study was supported by Health and Labor Sciences Research Grants of Japan and Grant-in-Aid for Scientific Research from the Japan Society for Promotion of Science.

Conflicts of Interest The authors declare no conflicts of interest.

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EMG Analysis of Human Inspiratory Muscle Resistance to Fatigue During Exercise

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Abstract

The aim of this study was to characterize the pattern of inspiratory muscle fatigue and to assess the resistance to fatigue of the diaphragm (D), parasternal (PS), sternocleidomastoid (SCM), and scalene (SC) muscles. Nine healthy, untrained male subjects participated in this study. Electromyographic activity (EMG) of D, PS, SCM, and SC was recorded during an incremental cycling test to exhaustion (workload of 1.0 W/kg with 0.5 W/kg increments every 5 min). The before-to-after exercise measurements of maximal inspiratory pressure (MIP) and EMG power spectrum changes were performed. The maximal inspiratory pressure declined about 8.1 % after exercise compared with that in the control condition (124.3 ± 8.5 vs. 114.2 ± 8.9 cmH_2O) ($P > 0.05$), whereas the peak magnitude of integrated electrical activity of D, PS, SCM, and SC during the post-exercise Müller maneuver was significantly greater in all subjects than that pre-exercise. The extent of inspiratory muscles fatigue was evaluated by analysis of a shift in centroid frequency (f_c) of EMG power spectrum. Exercise-induced D fatigue was present in three subjects and PS fatigue was another in two; whereas both D and PC fatigue were observed in four subjects. All subjects demonstrated a significant reduction in f_c of SCM and SC. Results indicate that early signs of the fatiguing process might be detected in the D, PS, SCM, and SC muscles during exercise to exhaustion. Fatigue of either D or PS muscles develops selectively or together during exhaustive exercise, depending on

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_29,

the recruitment pattern of respiratory muscles. Accessory inspiratory muscles of the neck are less resistant to fatigue compared with the D and PS muscles.

Keywords
Diaphragm • EMG • Exercise fatigue • Parasternal muscle • Scalene muscle • Sternocleidomastoid muscle

1 Introduction

Inspiratory muscle fatigue can develop during exhaustive high-intensity exercise in healthy subjects (Johnson et al. 1993; Mador et al. 1993; Babcock et al. 2002; Romer and Polkey 2008; Verges et al. 2006) and may become a limiting factor for exercise (Mador and Acevedo 1991; Martin et al. 1982; Perlovitch et al. 2007). Muscle fatigue can be defined as a loss in the capacity for developing force and/or velocity resulting from muscle activity under load, which is reversed by rest (NHLBI 1990). Nevertheless, initial signs of inspiratory muscle fatigue might be identified in spectral analysis of electromyographic (EMG) signals before a factual decrease in the muscle force. A classical surface EMG spectral variable applied to assess muscle functions is the centroid power frequency (Lindstrom et al. 1977; Aldrich et al. 1983). Shifts toward lower frequencies in respiratory muscles EMG spectra have been observed and, particularly, in the diaphragm during severe and prolonged inspiratory resistive breathing (Bellemare and Grassino 1982; Fitting et al. 1988; Segizbaeva and Aleksandrova 2009), near-maximal voluntary hyperpnea (Sieck et al. 1985), heavy muscular exercise (Hussain and Pardy 1985), and during circulatory shock (Hussain et al. 1988). Different accessory inspiratory muscles are recruited during exhaustive exercise to provide high levels of ventilatory requirements. The role of extra-diaphragmatic muscles increases during high inspiratory effort in relation to total respiratory output. It is likely that fatigue of extra-diaphragmatic muscles plays a significant role in exercise limitation (Perlovitch et al. 2007). It has been demonstrated that a female diaphragm is more resistant to fatigue relative to their male counterparts (Guenette et al. 2010). However, little is known about comparative resistance to exercise-induced inspiratory muscle fatigue of major and accessory muscles in healthy humans.

The aims of the present study were: (1) to investigate the effect of high-intensive incremental exercise to exhaustion on inspiratory muscle function, (2) to assess the resistance of the diaphragm (D), parasternal (PS), sternocleidomastoid (SCM), and scalene (SC) muscles to fatigue during exhaustive exercise, and (3) to analyze the individual changes in the centroid frequency of power spectrum before and after exercise test.

2 Methods

A local Committee on Human Research approved the protocol of the study. The study was performed in accordance with the ethical standards of the Helsinki Declaration for Human Experimentation. Each subject was familiarized with the experimental procedures and protocol, and gave informed consent to participate. Nine healthy, nonsmoking untrained male subjects participated in the study. All subjects had no history of cardio-respiratory diseases. All of them had the same daily schedule, meals, and the level of physical activity. Subjects were required to refrain from physical exercise for 2 days before the test, to refrain from drinking caffeinated beverages on the test day and to have a last meal at least 2 h prior to the test.

2.1 Pulmonary Function and Incremental Exercise Test

Pulmonary function test was performed with an ergospirometric-computerized device using a calibrated turbine for volume measurement (Schiller, Switzerland) in a sitting position. The forced expiratory volume in 1 s (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio, and peak expiratory flow (PEF) were recorded according to the recommendations of the ATS/ERS Statement (2002).

Exercise test was performed on an electronically braked cycle ergometer (Schiller, Switzerland) using a standard (5 + 1-min) incremental cycle exercise protocol. Subjects started with a 5-min period of pedaling with a workload of 1 W/kg at 60 cycles/min, which was followed by 1 min of rest. Then, workload was automatically increased by 0.5 W/kg every 5 min with 1-min resting period after each exercise bout until the subject was no longer be able to maintain the pedaling frequency at a constant level (not <70 per min). The subjects were strongly encouraged to cycle until exhaustion was reported.

2.2 Ventilatory Parameters

Minute ventilation and its components were measured continuously using an ergospirometric device (Schiller, Switzerland) during the incremental exercise test. Concentration of expired O_2 and CO_2 was measured breath-by-breath with the same device by using paramagnetic (O_2) and infrared absorption (CO_2) gas analyzers. These measures and flow signals were electronically integrated by a computerized system to yield 10-s averages of minute ventilation (V_E), respiratory duty cycle (T_I/T_T), tidal volume (V_T), respiratory rate (RR), oxygen consumption (VO_2), CO_2 production (VCO_2), gas exchange ratio (RER), end-tidal CO_2 pressure ($P_{ET}CO_2$), and end-tidal O_2 pressure ($P_{ET}O_2$). Calibrations were performed before each test. The heart rate was also measured and used to obtain the O_2-pulse. Arterial hemoglobin oxygen saturation (SaO_2) was monitored noninvasively using a pulse oximeter.

2.3 Maximal Inspiratory Pressure Measurements

Maximal inspiratory mouth pressure (MIP) was measured with a portable device (PowerBreath, UK) in accordance with the ATS/ERS Statement (2002). MIP was recorded at the mouth during a quasi-state short maximal inspiration against occluded airways (Müller's maneuver). The maneuver was performed at residual volume (RV) (Troosters et al. 2005). Each participant was instructed to expire slowly and completely as far as possible and then to perform a maximal inspiratory effort. The subjects were verbally encouraged by the operators to achieve a maximal result. To prevent closure of the glottis and to avoid significant pressure generation by the muscles of the cheek, a small leak was present in the equipment. The subjects performed a minimum of five maneuvers until two maximal pressure values were obtained which did not differ by more than 5 %; the higher of the two was chosen for analysis. The maneuvers were performed in the sitting position before and immediately after exercise test. For the sake of convenience, MIP was expressed in positive values.

2.4 Surface EMG Recording

Surface electromyograms (EMG) of the diaphragm (D), parasternal (PS), sternocleidomastoid (SCM), and scalene (SC) muscles were obtained with surface electrocardiographic electrodes 5 mm in diameter (ARBO, TYCO Healthcare Group LP, Germany). The EMG of D was recorded by electrodes applied to the skin on the anterior axillary line at the level of the eighth intercostal space (Verin et al. 2002). The EMG of SCM was obtained with electrodes positioned longitudinally over the middle third of the muscle on the right side of the neck, while the PS EMG was recorded with electrodes placed in the

second right intercostal space close to the sternum. The EMG of SC was obtained from electrodes placed in the posterior triangle of the neck (right side) at the level of the cricoid cartilage. The place was located during sniff maneuvers through palpation of the neck in the lower third of a line drawn between the middle of the mastoid process and the sternal notch. Within each electrode pair, the inter-electrode distance was <2 cm. Impedance was decreased by careful skin shaving and abrasion with alcohol. The wires connected to the electrodes were carefully secured with tape to minimize movement artifacts. All the EMGs were amplified and continuously recorded on a six-channel recorder (Biograph, Russia). EMGs were displayed and visualized simultaneously. All the data were stored on PC for future analysis. To quantify the EMG, the signals were filtered (10–1,000 kHz) and integrated on a moving-time-average basis with a time constant of 150 ms. The peak amplitude of integrated EMG was measured for each inspiratory muscle during valid Muller's maneuver before and after exercise test. The peak integrated EMG activity was measured in arbitrary units and then expressed as a percentage of the value, reached during Müller's maneuver before exercise test (control). A fast Fourier transform of EMG was used to compute the power spectral density. The power spectra were quantified in terms of the centroid frequency (f_c) (Hary et al. 1982). The f_c was measured in Hz and then expressed as a percentage of the value reached during inspiration before exercise test, taken for 100 % (control).

2.5 Data Analysis

Data are presented as mean values ± SE. To assess the development of respiratory muscle fatigue during exhaustive exercise, absolute values of maximal inspiratory pressures and centroid frequency of power spectra before and after exhaustive exercise were analyzed. Differences between post-exercise MIP as well as f_c were compared with data in the pre-exercise (control, taken for 100 %) with a paired *t*-test. All statistical analyses were performed using standard statistical software (Origin 6.1). $P < 0.05$ was considered statistically significant.

3 Results

3.1 Subject Characteristics and Pulmonary Function

The anthropometric characteristics and pulmonary function data of subjects are listed in Table 29.1. The intrasubject variability of basic descriptive characteristics was low. The subjects had absolute values for FVC, FEV_1, FEV_1/FVC, and PEF within normal limits.

3.2 Incremental Exercise Test

Table 29.2 summarizes resting and peak exercise ventilatory and metabolic measurements. The maximal work capacity was 223.8 ± 13.6 W. All subjects showed an increase in minute ventilation (V_E) (87.7 ± 5.3), achieved by increasing both tidal volume (V_T) and breathing frequency (f_b). They had a high absolute and relative maximal O_2 consumption and CO_2 production. There

Table 29.1 Baseline characteristics of the subjects

Anthropometrics	
Age, years	19.1 ± 0.3 (18–20)
Body height, cm	178.6 ± 1.6 (170–185)
Body weight, kg	77.6 ± 3.1 (67–91)
BMI, $kg \cdot m^{-2}$	24.9 ± 0.83 (20.1–28.1)
Pulmonary function test	
FVC, l	5.31 ± 0.3 (4.34–6.64)
FVC, %pred	98.6 ± 2.7 (84–121)
FEV_1, l	4.54 ± 0.26 (3.02–5.55)
FEV_1, %pred	98.7 ± 0.4 (86–115)
FEV_1/FVC, %	99.6 ± 0.12 (80–118)
PEF, l/s	8.72 ± 0.42 (6.9–9.86)
PEF, %pred	91.9 ± 3.2 (79–99)
HbO_2Sat, %	98.9 ± 0.4 (98–100)

Values are means ± SE (range)
BMI body mass index, *FVC* forced vital capacity, *FEV1* forced expired volume in 1 s, *PEF* peak expiratory flow, *HbO_2Sat* percutaneous O_2 saturation of hemoglobin in arterialized blood

Table 29.2 Rest and maximal incremental exercise data

Variables	Rest	Maximal exercise
W_{max}, Watt	–	223.8 ± 13.6 (153–253)
V_E, l/min	7.8 ± 1.7 (5.2–13.0)	87.7 ± 5.3 (56–123)
V_T, l	0.6 ± 0.1 (0.4–1.3)	2.7 ± 0.2 (2.4–3.7)
f_b, breaths/min	14.0 ± 1.9 (7.1–21.2)	32.3 ± 2.1 (23.0–44.2)
VO_2, l/min	0.269 ± 0.016 (0.2–0.352)	3.1 ± 0.2 (2.5–3.9)
VO_2, ml · kg^{-1} · min^{-1}	3.97 ± 0.39 (2.7–6.1)	41.2 ± 0.8 (34.2–52.7)
VCO_2, l/min	0.214 ± 0.012 (0.15–0.26)	3.290 ± 0.15 (2.73–4.03)
VCO_2, ml · kg^{-1} · min^{-1}	3.04 ± 0.29 (1.93–4.5)	41.85 ± 1.92 (34.64–54.4)
RER	0.78 ± 0.08 (0.67–0.88)	1.15 ± 0.13 (0.99–1.44)
HR, beats/min	74.1 ± 3.6 (56–86)	181.7 ± 4.9 (161–196)
O_2-pulse, ml/beat	3.6 ± 0.13 (2.3–4.8)	18.3 ± 1.3 (12.2–24.4)
$P_{ET}CO_2$, mmHg	32.02 ± 1.0 (28.5–35.1)	38.91 ± 2.16 (31.2–49.9)
$P_{ET}O_2$, mmHg	107.07 ± 2.50 (103.1–119.3)	114.27 ± 2.58 (101.3–122.2)
RWC_{170}, Wt	–	197.3 ± 21.8 (153–253)
RWC_{170}, Wt/kg	–	2.51 ± 0.3 (1.19–4.0)
AT, Watt	–	208.8 ± 12.4 (153–273)

Values are means ± SE and range
W_{max} maximal work performance, V_E minute ventilation, V_T tidal volume, f_b breathing frequency, VO_2 oxygen consumption, VCO_2 carbon dioxide production, *RER* respiratory exchange ratio, *HR* heart rate, O_2*-pulse* oxygen pulse, $P_{ET}CO_2$ end-tidal CO_2, $P_{ET}O_2$ end-tidal O_2, RWC_{170} work capacity at 170 beats/min, *AT* anaerobic threshold

were similar results of cardiovascular (HR_{max}) and O_2 delivery indices (O_2-$pulse_{max}$). The peak exercise values of these parameters reflect the middle fit level of volunteers. At peak exercise, all subjects reported less breathing then leg discomfort.

3.3 Respiratory Muscle Function and EMG Responses

We found a post-exercise decrease in maximal inspiratory pressure (MIP) (Fig. 29.1a). The drop in MIP was about 8.1 % on average ($P > 0.05$). Post-exercise values of MIP tended to decrease in seven out of the nine subjects. At the same time, the peak magnitude of integrated electrical activity of D, PS, SCM, and SC during post-exercise Müller's maneuver was significantly greater than that pre-exercise in all subjects. As illustrated in Fig. 29.1a and b, post-exercise Müller's maneuver produced greater-than-control ED, EPS, ESCM, and ESC amplitudes in order to provide lower values of MIP. This fact might be evidence of contractile, not central, inspiratory muscle fatigue after exhaustive exercise in normal humans.

EMG analysis demonstrated a significant increase in electrical activity of D, PS, SCM, and SC during incremental exercise (Fig. 29.2). To evaluate changes in the amount of fatigue between pre- and post-exercise tests, the before-to-after decrease in centroid frequency (f_c) was compared. Table 29.3 demonstrates the average absolute values of f_c in control and immediately after exhaustion. A shift toward a decrease in centroid frequency of power spectra after exhaustive exercise is a sign of inspiratory muscle fatigue development, too. To assess resistance to fatigue of different inspiratory muscles we analyzed centroid frequency changes. These data are presented in Fig. 29.3 and point to an individual pattern of f_c changes in D, PS, SCM, and SC after exercise relative to control taken for 100 %. Diaphragm fatigue alone was present in three (subjects №5, 6, 7), rib cage (PS) fatigue in two (subjects №2, 3) out of the nine subjects, whereas both D and PS fatigue was observed in four subjects. Figure 29.3 depicts a significant reduction in centroid frequency of SCM and SC in all subjects.

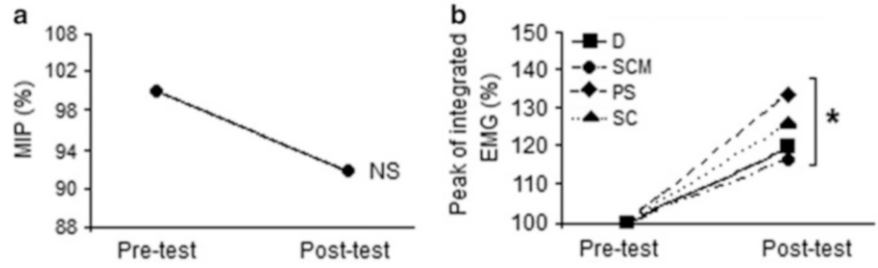

Fig. 29.1 (**a**) Change in mean maximal inspiratory pressure (MIP) after exhaustive exercise. MIP was expressed as a percentage of the maximal values achieved during Muller's maneuver before exercise test. *NS* non significant; (**b**) Mean peak amplitudes of the integrated EMG of the diaphragm (*D*), parasternal (*PS*), sternocleidomastoid (*SM*), and scalene (*SC*) muscles during Muller's maneuver after exhaustive exercise. Peak amplitude of EMGs was expressed as a percentage of the maximal values achieved during Muller's maneuver before exercise test. $*P < 0\,05$ in comparison to control values

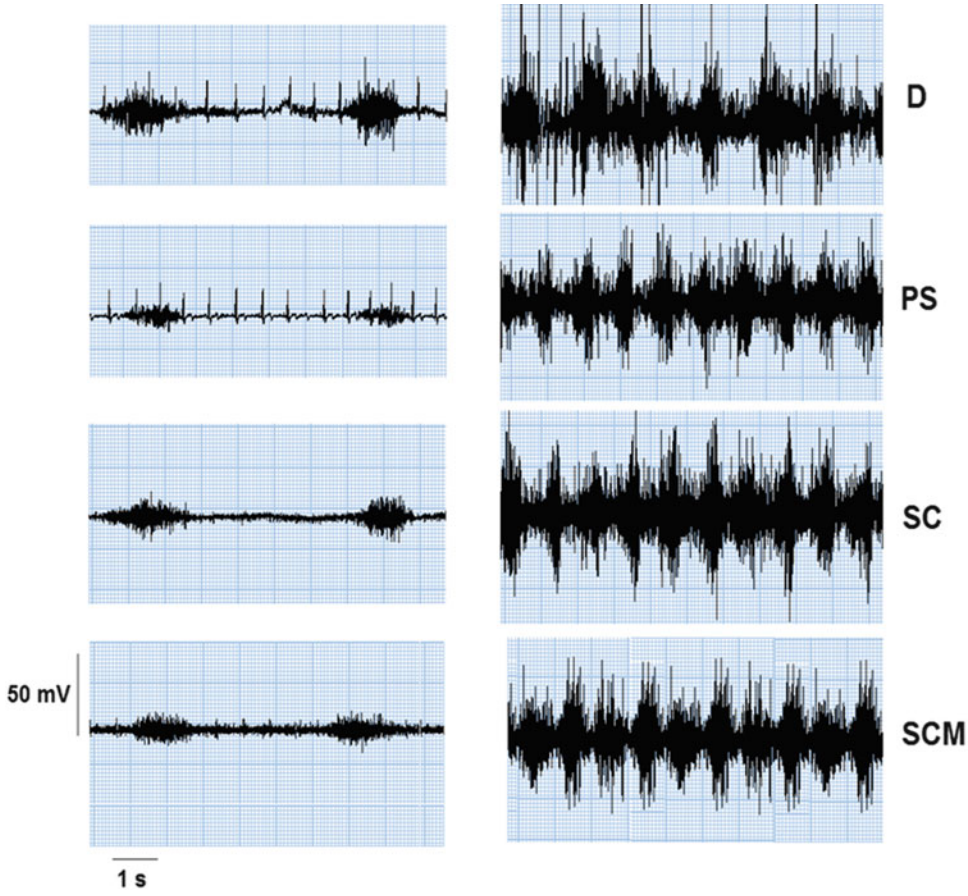

Fig. 29.2 Typical EMG recordings of the diaphragm (*D*), intercostal parasternal (*PS*), sternocleidomastoid (*SM*), and scalene (*SC*) muscles before exercise and during peak-exercise

Table 29.3 Strength and surface electromyographic parameters of inspiratory muscles before and after exercise

Variables	Pre-test	Post-test
MIP, cmH_2O	124.3 ± 8.5 (96–171)	114.2 ± 8.9 (78–158)
f_c D, Hz	160.1 ± 5.7	129 ± 5.2*
f_c PS, Hz	187.7 ± 5.6	161.1 ± 7.5
f_c SCM, Hz	146.3 ± 3.7	118.2 ± 5.7*
f_c SC, Hz	153.5 ± 4.4	133.7 ± 4.1*

Data are means ± SE and range
MIP maximal inspiratory pressure, f_c centroid frequency of diaphragm (*D*), parasternal (*PS*), sternocleidomastoid (*SCM*) and scalene (*SC*) muscles
*$P < 0.05$ for the pre- and post-test comparison

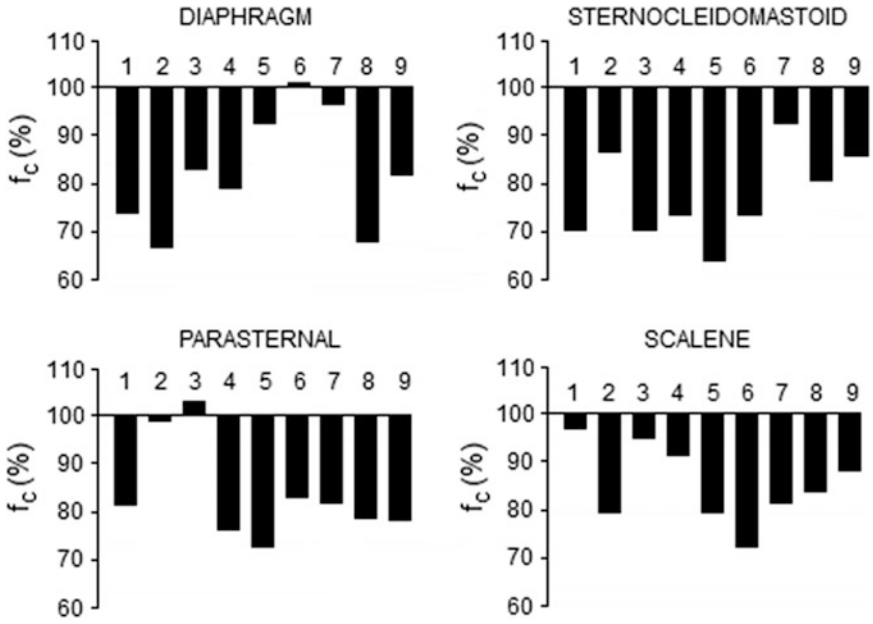

Fig. 29.3 Changes in centroid frequency (f_c) of the diaphragm (*D*), parasternal (*PS*), sternocleidomastoid (*SM*), and scalene (*SC*) muscles after exhaustive exercise in individual subjects (from 1 to 9). Centroid frequency of power spectra was expressed as a percentage of the mean values achieved before exercise test

4 Discussion

An increase in inspiratory muscle effort during high-intensive exercise occurs due to increased activation of the diaphragm and rib cage muscles as well as recruitment of accessory respiratory muscles, such as the sternocleidomastoid and scalene muscles. These muscles are responsible for the cranial displacement of the sternum and rib cage during inspiration. Like other skeletal muscles, all respiratory muscles might develop fatigue when contracting above a certain threshold of tension and time. The present study was designed to evaluate the resistance to fatigue of these major and accessory inspiratory muscles after high-intensive exhaustive exercise. It is known that the development of respiratory muscles fatigue may be predicted by changes in EMG power spectrum (Aldrich et al. 1983). Modifications of EMG occurring during fatiguing contractions can be characterized by a

decrease in the centroid frequency of the power spectrum (Aldrich et al. 1983; Schweitzer et al. 1979). To our knowledge, there are no reports that examine simultaneous EMG responses of major and accessory inspiratory muscles in normal humans during incremental exhaustion exercise. Our results of the EMG-analysis demonstrate consistent inspiratory muscle fatigue, which developed during intensive exercise. Interestingly, we found a different pattern of inspiratory muscle fatigue in individuals. A shift in f_c toward low frequencies pointed to the preferential fatigue of either D or PS inspiratory muscles, or both during high intensity exercise, which may reflect the contribution of these muscles to increased ventilatory requirements. These observations are in line with studies in which the measurements of P_{di} and P_{es} were done. It was shown that rib cage muscles are preferentially fatigued during inspiratory resistive loading (Hershenson et al. 1989). Moreover, it has been previously observed that fatigue of either D or PS and SCM muscles can occur independently during inspiratory resistive breathing according to voluntary thoracic or abdominal pattern of breathing (Fitting et al. 1988). This suggestion is supported by the fact that resistive breathing with diaphragmatic pattern results in D fatigue, whereas rib cage breathing pattern produces PS and SCM fatigue (Fitting et al. 1988). The results of the present study support this assumption and demonstrate different inspiratory muscle resistance to fatigue during exhaustive exercise without resistive load, depending on the preferential contribution of D or thoracic muscles to inspiratory effort. All subjects demonstrated a significant reduction in f_c of SCM and SC in the present study. This suggests that accessory inspiratory muscles of the neck are less resistant to fatigue during intensive contractions compared with D and PS muscles. The difference in resistance of major and accessory inspiratory muscles to fatigue might be related with muscle fiber composition, neuromuscular characteristics, high-energy metabolite stores, buffering capacity, ionic regulation, capillarization, and mitochondrial density (Bogdanis 2012). On the other hand, it seems possible that improvement of inspiratory muscle coordination and thus efficiency may increase resistance to fatigue and delay its development.

We conclude that exhaustive cycling exercise results in a decrease in maximal inspiratory pressure despite increases in the integrated EMG of D, PS, SCM, and SC muscles, suggesting that contractile inspiratory muscle fatigue developed. It appears likely that fatigue of either diaphragm or rib cage muscles may develop selectively or in both muscles during exhaustive exercise, depending on the recruitment pattern of respiratory muscles. Diaphragm and parasternal muscles have a greater resistance to exercise-induced muscles fatigue than accessory inspiratory muscles of the neck.

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

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Assessment of Temporomandibular Joint Disease

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Abstract

The diagnosis of temporomandibular joint (TMJ) disorders consists of clinical (Reaserch Diagnostic Criteria for Temporomandibular Disorders, RDC/TMD) and additional (computer tomography, CT or magnetic resonance imaging, and MRI) examinations. Due to the growing knowledge of pathologic changes within the TMJ, the researches become more aware of the difficulty in detection the early symptoms of disorders using conventional examination. Therefore, it is now expected that the collected samples of synovial fluid, serum, or urine samples could enable easier identification of inflammatory process course, and degenerative cartilage changes state.

Keywords

Bone turnover markers • Cytokines • Internal derangements • Osteoarthritis • Temporomandibular joint

1 Introduction

Temporomandibular joint (TMJ) is a synovial joint with the presence of an articular disc. Disorders affecting the TMJ encompass the intra-articular positional changes and structural changes (Stegenga 2010; Dworkin and Le Resche 1992). Positional changes in the joint apply to the disc and to condyle relation in the articular fossae and are called internal derangements (ID). Depending on the disc placement in the maximal intercuspal position and during mandibular movement, the disc displacement could be with or without reduction. This kind of disturbance impairs the function of the masticatory system. They are mostly characterized by pain in the TMJ and surrounding structures, the presence of joint sounds and disturbed or limited mandibular

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_30,

movements. Structural changes of TMJ include arthritic and growth disorders. The arthritic disorders comprise inflammation, degeneration, and deformations in the joint. The suggested mechanisms of TMJ disease are mechanical injury, hypoxia-reperfusion injury, and neurogenic inflammation (Cairns 2010; Milam and Schmitz 1995; Stegenga et al. 1989). It has been revealed that positional changes in the TMJ could be the cause or result of structural changes (Sylvester et al. 2011; Stegenga et al. 1989). This fact has essential treatment implication, since antiinflammatory treatment should be considered in positional disturbances to prevent later structural degenerative changes of TMJ.

Procedures described in the Research Diagnostic Criteria for Temporomandibular Disorders (RDC/TMD) are generally accepted for clinical diagnosis of TMJ disorders (Dworkin and Le Resche 1992). Additional diagnostic methods comprise mainly imaging modalities, using panoramic radiography, magnetic resonance imaging (MRI), and computed tomography (CT) (Ahmad et al. 2009). However, inflammatory and degenerative changes begin in the fibrous cartilage, which is not detectable by imaging (Milam and Schmitz 1995). Attempts have been made to use the biomarkers of inflammation and bone turnover for early diagnosis of various TMJ conditions. There are various sources of samples for assaying such markers, e.g., synovial fluid, serum or plasma, or urine. The TMJ tissue responds to etiological factors by producing several inflammatory mediators (interleukins, cytokines), releasing neuropeptides (substance P, calcitonin gene-related peptide), matrix-metalloproteinases, and inhibitors of tissue inflammatory mediators (Fernandes et al. 2002; Milam and Schmitz 1995). Stimulation of TMJ nociceptors increases the release of neuropeptides. Inflammatory mediators and/or local hypoxia not only initiate the remodeling or degenerative changes in the joint, but also lead to generalized pain (Cairns 2010). It is suggested that pain intensity in temporomandibular disorders correlates with expression of calcitonin gene-related peptide (CGRP) and substance P. The concepts of the pathomechanism of osteoarthrosis indicate a role of inflammatory markers such as cytokines (interleukin-1, IL-1, or tumor necrosis factor-α, TNF-α) in cartilage matrix degradation and tissue net loss (Stegenga et al. 1989). Pathological changes are characterized by deterioration and abrasion of articular cartilage and a thickening remodeling of the underlying bone.

The structure of TMJ, particularly of the disc, has been described in light microscopy as being composed of collagen and elastic fibers with chondroid cells (Singh and Detamore 2009; Keene et al. 1987). TMJ structures are mainly covered with fibrous cartilage. In bone and soft tissues, type I collagen is most abundant, with type III collagen co-distributed with it. Synovial joints adapt to the functional forces by remodeling. However, when loading exceeds the adaptive capacity of the joint, pathological changes begin.

2 Temporomandibular Joint Inflammatory Markers

TMJ internal derangements and osteoarthrosis are associated with inflammatory processes in the synovial membrane and articular cartilage (Fernandes et al. 2002). It has been revealed that an excess amount of inflammatory mediators in the synovial fluid of TMJ as well as their characteristics, balance, and receptors play an important role (Kaneyama et al. 2002, 2005; Fang et al. 1999; Kubota et al. 1997, 1998a, b). Cytokines, such as IL-1 and TNF-α, can cause cartilage degradation through upregulation of metalloproteinases (MMP) gene expression and a decrease in the chondrocyte compensatory synthesis pathways. In TMJ, synovium of the patients with osteoarthrosis has a higher concentration of cytokines and protein compared with healthy subjects and imbalance between cytokines and their soluble receptors is observed (Kaneyama et al. 2005). The ratio between cytokines and their receptors can influence the homeostasis and prognosis of pathological processes in TMD patients.

Increased levels of cytokines (IL-1, IL-6) and active forms of MMP suggest cartilage

degradation that has been reported not only in osteoarthrosis but also in patients with TMJ internal derangements with a disc displacement without reduction (Kubota et al. 1998a, b). Another study has revealed that increased concentration of IL-6 in synovial fluid can reflect degenerative changes of the condyle (Kaneyama et al. 2002). It is also suggested that IL-1 and stromelysin (MMP3) can be the markers of early bone deterioration, which are not detected by radiograph imaging (Kubota et al. 1997). At the bone degrading stage of internal derangements, IL-1 comes from both synovial macrophages but also from chondrocytes or fibroblasts of the articular cartilage, which suggests the possibility of detection early osteoarthritic changes.

Inflammatory markers, such as TNF-α, IL-1α, IL-1β, IL-6, IL-8, and various kinds of MMPs, should be investigated not only in terms of their concentrations but also of their ratio to specific receptors and other mediators (Kaneyama et al. 2005), which is essential for treatment prognosis.

3 Markers of Bone Turnover

In general medicine, biomechanical markers of bone turnover are used in cases of osteoporosis, bone tumors, osteonecrosis, osteoarthrosis, or rheumatoid arthritis RA (Garnero 2009; Wollheim 2002; DeGroot et al. 2002). They reflect both synthesis and resorption of cartilage and bone. Their concentrations are measured in the serum, urine, and synovial fluid. The markers are derived from fragments of collagen, and enzymes and proteins released into the blood during metabolic activity of chondro- and osteoblasts as well as chodro- and osteoclasts. These markers are sensitive indicators of early metabolic disturbances in cartilage and bone. Bone turnover markers allowed for the assessment of the rate of bone formation and resorption processes.

Difficulties in using the bone turnover markers in TMJ disease are due to the specific structure of the joint and its small size. The concentration of the bone turnover markers is a sum of all metabolic processes in osteoarticular system. The TMJ structures are mostly covered with fibrous rather than hyaline cartilage, like the majority of human joints. Type II collagen is the major type of collagen in the hyaline cartilage matrix. The fibrous cartilage contains mainly type I collagen, which also accounts for 90 % of the organic part of bones. An increased concentration of CTx-I (C-terminal cross-linked telopeptides of collagen alpha chains type I) reflects the loss of both TMJ fibrous cartilage and bones. The CTx-II concentration (C-terminal cross-linked telopeptid alpha chains of collagen type II) can only confirm the loss of hyaline cartilage in degenerative changes in the joints (Reijman et al. 2004; Garnero et al. 2003; Young-Min et al. 2001), which is of no use in TMJ cases. The evaluation of the TMJ cartilage must be done using different tests. Tanimoto et al. (2004) used pyridinoline and deoxypyridinoline markers in the diagnosis of TMJ osteoarthrosis. These proteins form intermolecular bonds in mature forms of collagen. They are not metabolized in the body, but completely eliminated by kidneys. These markers are present in the cartilage of the TMJ, so it is possible to assess the relationship between markers' concentration and osteoarthrosis severity. Tanimoto et al. (2004) have selected 12 patients with TMJ osteoarthrosis for the study. Urine samples were collected and then pyridionline concentration and the ratio of pyridinoline to deoxypyridinoline (Pyr/dPyr) were measured by high performance liquid chromatography. The results were compared with the control group and correlated with the radiographic findings of TMJ images. There was a significant increase in the concentration of pyridinoline and the Pyr/dPyr ratio in patients with osteoarthrosis. There was, however, no relationship between the test results and osteoarthrosis progress visible on radiographs. The difficulty in detecting early radiographic bony changes may lie in the discrepancy between the time needed for the changes to be visible in images and the progress of an intra-articular pathology; the latter advancing much faster. Tanimoto et al. (2004) suggested the usefulness of using the bone turnover markers in conjunction

with a conventional image analysis for the differential diagnosis of TMJ osteoarthrosis. Unlike conventional radiological examinations, with a limited use at early stages of osteoarthrosis and their retrospective character, the markers could provide early diagnosis and the assessment of therapeutic effects. Further studies on large groups of patients are necessary to establish a reliable marker reflecting the pathological changes within the TMJ cartilage and to determine the appropriate source of biological material for research (serum, urine, synovial fluid).

4 Summary

The multifactorial etiology of temporomandibular disorders compelled clinicians to apply a comprehensive diagnosis and noninvasive, conservative treatment (Donovan et al. 2012). In many cases, this is just the treatment of symptoms. Therefore, it is essential to investigate the underlying pathogenesis of such disorders to develop tissue-targeted therapies, to stop or even reverse the pathologic process. However, in causal therapy, precise and early diagnosis is very important. It seems essential to seek for markers specific for the temporomandibular joint pathology. Compared with classical radiological methods, the markers may be key for early detection of pathology and may serve as a source of information about therapeutic effects. Taking into consideration the cartilage and bone degradation, biochemical markers of bone turnover seem to meet the expectations, especially in diagnosis of osteoporosis, bone tumors, osteonecrosis, osteoarthritis, and rheumatoid arthritis. To-date, unfortunately, these markers are sensitive but not specific. Since they regard all bone remodeling, it is difficult to determine their reflecting but a temporomandibular joint process. The same problem arises when interpreting the concentration of inflammation indicators in the blood. It has so far been found that only plasma bradykinin correlates with its synovial fluid concentration, which however has no relation to pain intensity. Specific information about the temporomandibular joint inflammation could be obtained from a biochemical examination of aspirated synovial fluid. The concentration of neuropeptides, cytokines, leukotrienes, prostaglandins, and catabolic products identified in temporomandibular fluid correlates with joint pain and with the presence of surface lesions observed within the joint. The bone turnover markers, along with the assessment of inflammatory markers and imaging analysis, seems to provide the information on the severity of degenerative changes in the joint structures and on the inflammatory process in the course of osteoarthritis.

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

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Spirometry Day: A Means to Enhance Social Knowledge on Respiratory Diseases

31

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Abstract

The chapter presents the results of pulmonary function tests conducted as part of the Polish Spirometry Day of 2011, an initiative aimed at increasing the awareness of causes, symptoms, and delayed effects of common respiratory diseases, in particular of bronchial asthma and chronic obstructive pulmonary disease, and at demonstrating the role of regular examinations, especially in higher risk groups. The results show that there was a relatively substantial group of persons, 11.2 % of the population sample studied, not being aware of a respiratory disease they had. Furthermore, the results show that quite often, 12.4–16.0 % of the population studied, obstruction was diagnosed in persons who did not have any spirometry tests done before, despite some respiratory symptoms that should raise the attention of general practitioners to perform such tests.

Keywords

Bronchial asthma • Chronic obstructive pulmonary disease • Pulmonary function • Respiratory diseases • Screening tests

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1 Introduction

Chronic obstructive pulmonary disease (COPD) is one of the major causes of human morbidity and mortality. According to WHO, COPD is the fourth main cause of death worldwide. One of the reasons for the problem with lung diseases is late detection (Mannino et al. 2000). An impact on disease progress may also be caused by lack of social awareness of its aetiology. Risk factors for COPD are both host factors (genotype) and environmental exposures. Development of the disease is accelerated by tobacco smoking, ambient air pollution, and occupational exposure to

M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_31,

dust and different chemicals (Romain et al. 2001). Numerous studies (Anderson et al. 2011; Canova et al. 2012; Colais et al. 2012; de Marco et al. 2011; Sousa et al. 2012) have confirmed that exposure to traffic air pollution affects human health, causing severe lung functional symptoms. The results of a cohort study conducted by Andersen et al. (2011) in Denmark on a group of 57,000 patients demonstrate that living close to busy roads increases the number of individuals suffering from COPD.

Similar conclusions are drawn by Lindgren et al. (2009) who have shown that residents living within 100 m from a road with traffic density over 10 cars/min, compared with no heavy traffic road at the distance, is associated with a higher incidence of asthma and COPD (OR = 1.40; 95 % CI = 1.04–1.89 and OR = 1.64, 95 % CI = 1.11–2.40 respectively). Badyda et al. (2013) have shown that the risk of obstruction among non-smoking persons living close to busy roads in cities is visibly higher than that among rural area residents (OR = 4.35; 95 % CI = 2.57–7.35 or OR = 3.16; 95 % CI = 1.09–9.16 for different cities). In the studies conducted by Peacock et al. (2011) short-term exposure to air pollutants such as nitrogen dioxide, ozone, sulphur dioxide, and to particulate matter (PM_{10}) contributes to exacerbation of COPD symptoms.

Many other studies (Corrêa et al. 2009; Lindstrom et al. 2001) have confirmed that cigarette smoking contributes to respiratory illnesses, decreasing FEV_1, and increasing death rates among smokers. Inhalation of particulate and gases associated with passive smoking exposure may also induce COPD and other respiratory symptoms (Leuenberger et al. 1994; Jaakkola and Jaakkola 2012). There is also evidence (Prescott et al. 1999) that the risk of developing COPD is highly related to low socioeconomic status.

One of the aims of creating Global Initiative for Chronic Obstructive Lung Disease (GOLD) was to prepare a global strategy for the COPD diagnosis, management, and prevention. Romain et al. (2001) pointed out that an essential aspect of public healthcare of COPD is to educate patients and physicians that breathlessness, chronic cough, and sputum production are not trivial symptoms and should be associated with disease recognition. Reduction of the risk factors should be focused on smoking cessation, which is the most effective and cost-effective way of minimizing the development and progress of COPD.

An important public health perspective, as underlined by Maio et al. (2012), is an early detection of airway obstruction in a large number of residents. To assess the possibility of achieving this goal, Fuller et al. (2012) designed a prospective study aimed at checking whether spirometry screening tests can be performed by pharmacists. The objective of the study focused on the reproducibility of spirometry results the accuracy of interpretation, and the improvement in the enrolment into smoking cessation programs. A group of 185 patients participated in the study. The study demonstrates that lung function tests can be implemented in a community pharmacy chain (99 % of the tests were judged acceptable and 90 % demonstrated reproducible results). Moreover, Sims and Price (2012) underline the problem of incorrect diagnoses of pulmonary diseases. Spirometry test, as it was pointed out, is an essential tool for general practitioners for differencing COPD from asthma.

Results of another study, conducted by Horie et al. (2011), emphasise that early COPD detection, is essential for preventing the progress and exacerbations of the illness. The researchers have shown that spirometry screening tests are an easy way to early-stage COPD detection. A study of Zielinski and Bednarek (2001), on over 11,000 subjects, positively evaluated the effectiveness of an early COPD detection in a high-risk population (tobacco smokers over 39 years old). Almost 1 quarter of the patients showed features of obstruction. A moderate level was found among 9.6 % of patients, while severe among 5.2 %. The conclusion of the paper by Zielinski et al. (2006) focuses on multidisciplinarity of healthcare teams. Researchers recommend concentration of the efforts at the global, national and local levels in order to reduce the burden of COPD.

In order to enable a large number of persons to have access to diagnostic screening tests and to enhance social awareness associated with tobacco use and with the effects of ambient air pollution as the prime causes of pulmonary diseases, there is a great need of performing annually and worldwide public actions such as the Polish Spirometry Day 2011 or World Spirometry Day 2012.

2 Methods

2.1 Material

The study was approved by the Ethics Committee of the Military Institute of Medicine in Warsaw, Poland. The study was completed on October 14th, 2011, with the participation of persons who signed up to medical units and of the institutions which declared cooperation within the Polish Spirometry Day. The tests were free of charge for the patients. A total of 4,088 test results from persons living in 490 different locations (large urban areas, smaller cities, and rural areas) were received. The tests which were incomplete, or contained uncertain, or did not meet technical correctness specified by ERS and ATS were excluded from further analysis. In the end, the analysis covered 2,881 pulmonary function tests.

2.2 Examination

Examinations were conducted in 256 medical centres and other institutions equipped with Easy One (AeroMedika, Warsaw, Poland), Spirolab (Ronomed, Wroclaw, Poland), MES 500 and MES 1000 (MES, Cracow, Poland) spirometers. In addition, a specialized questionnaire was used to help disclose the persons at risk or already suffering from a disease, but not aware of it yet. The scheme of the examination was as follows:

- Information about the aim of the examination and lack of its harmful impact on the human body;
- Self-reported survey – a questionnaire including the following: information about the place of living (locality, floor, distance to main roads, period of residence, etc.) and its conditions (heating method, presence of gas cookers, other emitters of air pollutants), smoking habit and its intensity, passive smoking, exposure to harmful factors, current pulmonary diseases, presence of symptoms that might have to do with respiratory disease, allergies, and anthropometric data;
- Objective research – pulmonary function test carried out in the sitting position, after a few-minute time given to adapt to the new breathing conditions. Several flow-volume curves were recorded, until the repeatability in accord with the American Thoracic Society (ATS) criteria was achieved, i.e., 3 measurements not varying by more than 5 %. The test results included the following parameters that are commonly used to assess ventilation:
  - FVC (forced vital capacity);
  - FEV_1 (forced expiratory volume during the first second of expiration);
  - FEV_1/FVC ratio – percentage indicator of the relation of FEV_1 to the present FVC. According to GOLD guidelines, obstruction was identified in individuals with FEV_1/FVC below 70 %.

The values expressed in litres were converted into predicted values according to the commonly used ERS/ECCS standards (Quanjer et al. 1993). Statistical analysis was conducted using Statistica 9.1 software. The Shapiro-Wilk test was used to assess distribution of variables, which were then compared with one-way ANOVA or Kruskal-Wallis test as required. A $p < 0.05$ was considered statistically significant. A test for differences between two structure indicators was used to compare percentage values.

3 Results

Airway obstruction was found in 357 individuals, which represents 12.4 % of the subjects investigated. Relatively low average pseudo-Tiffeneau factor (89.4 ± 16.9 %) and FEV_1 (93.1 ± 18.8 %) were found in the sample analyzed (Table 31.1).

Table 31.1 Basic spirometric variables %predicted values (n = 2,881)

Variable	Mean ± SD	Median	Lower-upper quartile
FVC	94.1 ± 19.6	96.0	82–108
FEV_1	93.1 ± 18.8	94.0	82–106
FEV_1/FVC	89.4 ± 16.9	89.7	78–101

Table 31.2 Persons with chronic pulmonary symptoms, examined with spirometry for the first time ever and showing evidence of obstruction

Chronic pulmonary symptom	Percentage
Dyspnea	12.8
Cough	12.0
Cough with expectoration	14.2
Wheezing while breathing	16.4

A large number of 1,860 individuals performed spirometry tests for the first time in their life. Among them, obstruction was found in 195 (10.5 %) cases. In the group of 944 persons who had been examined before, obstruction was found in 156 (15.7 %).

An important finding concerns the number of persons declaring a former diagnosis of a respiratory disease that has not been examined or confirmed with a spirometry test. There were 68 such persons who declared the diagnosis of asthma, 20 of COPD, and 78 of chronic bronchitis.

The study indicates a large discrepancy between the number of persons who declare a former diagnosis of respiratory diseases and the actual number of those who really suffer from a disease. Three hundred and eight (10.7 %) persons declared being diagnosed with asthma and/or chronic obstructive pulmonary disease in the past. Spirometry results demonstrated that only 42.2 % of the persons who declared being diagnosed with COPD, actually showed the features of obstruction. Either the statements were contrary to the facts or the diagnosis was incorrect, since the course of disease is always connected with bronchial obstruction. Among those who declared having asthma (n = 244), spirometry showed obstruction in 41 persons. Thus, the remaining 203 persons (83.2 %) did not show any features of obstruction. That may actually be possible, since only about 25 % of asthma sufferers show obstruction. Among persons with chronic bronchitis (n = 18), only were three cases of obstruction found. It should, however, be noted that persons with bronchitis, but without evidence of obstruction, are at potential risk, as chronic bronchitis may underlie the development of COPD.

Considering all persons who did not declare the diagnosis of asthma or COPD (n = 2,572), obstruction was observed in 289 (11.2 %) of them. Although these results may not necessarily be generalized for the whole population, they do show that a large percentage of persons who describe themselves as 'healthy' have features of obstruction in functional tests. Thus, a significant part of the society is not aware of a progressing respiratory disease.

There were some other respiratory complaints in persons who did not show obstruction in spirometry. Dyspnea was declared by 869 (34.4 %), dry cough by 952 (37.7 %), chronic cough with expectoration by 841 (33.3 %), and wheezing by 615 (24.4 %) persons. Among the 357 patients in whom airway obstruction was confirmed, the symptoms above listed occurred significantly more often: dyspnea (49.0 %), dry cough (45.1 %), cough with expectoration (47.5 %), and wheezing (37.9 %). It is worth noting that a substantial number of persons with one or more of these symptoms have never undergone spirometry testing, although in many of them obstruction was indicated (Table 31.2).

Differences in respiratory variables also were analyzed according to the place of residence. The study included 363 inhabitants of rural areas, 1,019 of small towns, and 1,536 residents of cities (>100,000 inhabitants). There were significant differences in the distributions of FEV_1 and FEV_1/FVC between some of the groups (details in Table 31.3). The highest percentages of predicted values were noted among the inhabitants of rural area. These values were lower in the residents of cities, and were the lowest among inhabitants of towns. A similar relationship was observed with respect to the percentages of persons with obstruction: 27 (8.3 %) individuals in

Table 31.3 Spirometric variables in city (≥100,000 inhabitants) and town (≤100,000 inhabitants) residents

Variable	Cities	Towns	Rural areas
FEV_1	94.3 ± 17.6	90.5 ± 19.7*	95.2 ± 20.4***
FVC	94.2 ± 18.5	93.8 ± 21.3	94.6 ± 19.1
FEV_1/FVC	89.4 ± 16.5	88.5 ± 17.7	92.5 ± 16.4**, ***

All values are means ± SD of % predicted
*p < 0.001 vs. cities; **p < 0.01 vs. cities; and ***p < 0.001 vs. towns

Table 31.4 Spirometric variables in smokers vs. non-smokers

Variable	Smokers	Non-smokers
FEV_1	92.7 ± 19.5	93.2 ± 18.4
FVC	93.5 ± 19.7	94.4 ± 19.5
FEV_1/FVC	87.4 ± 16.8*	90.5 ± 16.9

All values are means ± SD of % predicted
*p < 0.001

rural areas, 194 (12.6 %) in cities, and 136 (13.4 %) in towns.

There were 980 tobacco smokers in the cohort studied. Obstruction was found in 148 (34.0 %) of them. In the non-smokers (n = 1,901), there were 209 (11.0 %) cases of obstruction. We found a significant decline in FEV_1/FVC, but surprisingly, not in FEV_1 in smokers compared with non-smokers (Table 31.4).

Analysis of risk assessment shows that the risk of obstruction, depending on the severity of tobacco smoking, was higher among smokers. A simple logistic regression model, taking into account only the intercept and the variable smoking, indicates that smoking increases the risk of obstruction 1.6 times.

4 Discussion

The study demonstrates that a significant percentage of persons with chronic respiratory symptoms have never spirometry performed, despite obvious indications. Depending on the symptom, this percentage ranged from 21.7 % for wheezing to 37.3 % for cough. Moreover, in 12.0–16.4 % of patients experiencing symptoms, the presence of bronchial obstruction was shown. Each of these individuals received a letter to his general practitioner with a request to implement further diagnosis and treatment.

Mild obstruction was observed among 16.8 % persons declaring former diagnosis of asthma, which indicates that the majority of these persons had a mild form of disease. An important problem was observed in the persons declaring diagnosis of COPD, where obstruction was determined in only 42.2 % of them. Since almost 60 % of persons described themselves as patients with COPD, the criteria for the diagnosis of COPD were not met. It can be assumed that either the statements were incorrect or the diagnosis was false, which may have influenced the therapeutic management of these persons. No data on the methods of treatment were collected in the present study, which seems a notable issue to be considered in subsequent actions.

Considering that an essential objective of the Spirometry Day was to raise awareness of the factors causing respiratory diseases (Zielinski et al. 2006), foremost of tobacco smoking as a key factor, the organizers hoped that smokers would be coming in numbers to take advantage of the possibility of being examined. They were, however, represented in the group investigated in a percentage (34 %) similar to that existing in the general population (32 %).

Interestingly, significant differences in FEV_1 and FEV_1/FVC were found depending on the place of residence. The difference between inhabitants of rural areas and large cities concerning the presence of obstruction has been reported previously (Badyda et al. 2013). In the present study, however, the highest percentage of obstruction and the lowest values of spirometric variables were found in residents of towns, with population less than 100,000. This could results from:

- mode of thermal energy production that is decentralized in small towns and often uses fuel of unknown origin;

- problems with road transport, due particularly to lack of ring roads in towns situated upon major transport routes with heavy traffic;
- specific conditions of dispersion of air pollutants;
- general standard of life, access to health care, etc.

This phenomenon definitely needs further research. From this perspective, it is also worth paying attention to increased efficiency of environmental quality monitoring and effective implementation of environmental policies, as mentioned by Sauer et al. (2012). With regard to the upward trend in the incidence of COPD (Mannino and Buist 2007) and considering that both direct and indirect medical costs increase with increasing severity and acuteness of disease, regular monitoring seems definitely justified.

We conclude that spirometric screening helps detect previously undiagnosed cases of bronchial asthma or COPD, and helps verify false diagnosis. The screening makes it possible to implement appropriate treatment and reduces financial expenses.

Acknowledgements Polish Spirometry Day 2011 was organized under the patronage of: Polish Society of Allergology, Institute of Tuberculosis and Lung Diseases, Military Institute of Medicine, and National Health Fund.

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

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Lung Function in Divers

32

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Abstract

Correct lung function is indispensible to perform work underwater. Thus, spirometric tests of lung function remain an important element in the process of selecting candidates for professional diving. Studies conducted in the population of divers identified the phenomenon called 'large lungs', which is often associated with spirometric indices characteristic of obstructive impairment of lung function. This study investigated selected parameters of lung function in the population of divers and candidates for professional divers. Fifty two male subjects were examined as part of the selection process. Basic spirometric tests: forced expiratory volume in 1 s (FEV1; dm^3), forced vital capacity (FVC; dm^3), forced expiratory flow in the range 25–75 % of FVC (FEF25-75; $dm^3\ s^{-1}$), and FEV1/FVC (%) were compared with compared with the predicted reference values estimated by the European Coal and Steel Community. The results demonstrate differences in FVC and FEF25-75 in divers, which may correspond to functional hyperinflation. The effects of 'large lungs' observed in divers, if persisting for an extended period of time, may lead to lung ventilation impairment of the obstructive type.

Keywords

Divers • Hyperinflation • Forced vital capacity (FVC) • Forced expiratory flow (FEF) • Large lungs • Lung function • Spirometry

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_32,

1 Introduction

Effective functioning of the respiratory system is one of the most important elements of human physiology, especially in divers, in whom it has a considerable influence on the ability to perform useful work underwater. It is commonly known that any breathing resistance decreases the comfort of individuals working in aquatic environments (Vail 1971). Analysis of life function parameters will be useful to characterize this particular professional group against other groups in the society. This is an important issue as the majority of available medical literature points to the phenomenon called 'large lungs, which is often associated with spirometric indices characteristic of lung ventilation impairment of the obstructive type (Skogstad et al. 2000, 2002; Watt 1985; Davey et al. 1984; Crosbie et al. 1977, 1979). Any possible deviations observed while performing spirometric tests gain special importance in the aspect of legally regulated requirements for work as a professional diver. In the Polish Armed Forces spirometry has been routinely conducted for many years, while in the civilian environment an obligation to perform such tests was imposed in 2008 (Regulation of the Minister of Health 2007).

The aim of the present study was to perform a descriptive analysis of selected lung function parameters in divers and candidates for professional divers. The analysis was carried out on the basis of medical tests which are compulsory for all candidates willing to work as professional divers. The results were then compared with those obtained by other researchers dealing with the above subject matter.

2 Methods

2.1 Subjects

The study was approved by the Ethics Committee of the Military Institute of Medicine in Warsaw, Poland. The study population consisted of 52 men, divers, and candidates for professional divers, who were required to be medically examined before they were declared fit to dive. The group consisted of 42 certified professional divers ($n = 42$) and ten candidates for professional divers ($n = 10$). All candidates for professional divers had some diving experience in the field of recreational diving, confirmed by at least a basic level certificate issued by a diving federation. The study population consisted of individuals aged 20–49. There was a minor difference between the mean (31.3 years) and the median (30.5 years), causing a slight skewness of the age distribution. Approximately 80 % of the study population consisted of divers and candidates for professional divers were 20–38 years old. The distribution of body weight in the study group was 61–114 kg. There were no significant differences between the mean (83.5 kg) and the median value (84 kg). Approximately 80 % of the study population consisted of divers and candidates for professional divers with body weight of 70–95 kg. Height of the studied individuals was 167–195 cm; the mean value was 180.2 cm, and the median value 180.0 cm. The distribution of height in the study population was uniform; approximately 80 % of the group consisted of divers and candidates for professional divers were 168–190 cm tall. Body mass index (BMI) was 19.9–31.6 $kg \cdot m^{-2}$. The mean BMI amounted to 25.7 $kg \cdot m^{-2}$, and the median value was 25.0 $kg \cdot m^{-2}$. Approximately 80 % of the study population consisted of divers and candidates for professional divers had BMI close to normal, i.e., 20–28 $kg \cdot m^{-2}$.

2.2 Spirometry

The study was based on spirometry performed in 2010–2011. The measurements were taken by the same spirometer and the same operator. Lung function measurements were made with a SpiroUSB spirometer (Cardinal Health, Dublin, OH) and Spida-5 software (CareFusion Health, Basingstoke, UK). In accordance with generally accepted principles, the test was performed in a sitting position. Each subject was carefully instructed before the test, and each was informed that in case of any problems with proper performance, the test would be repeated, which is of no

health consequences. This information has a positive psychological influence on the quality of measurements (Gondorowicz and Siergiejko 2004), especially in subjects who have their lung function measured for the first time, are uncertain about the results, or for whom the results may have important legal implications. The spirometer was calibrated before each measurement according to the manufacturer's recommendations.

2.3 Case Definitions, Statistics

Spirometric indices were standardized and compared with predicted reference values according to the European Coal and Steel Community (ECSC) guidelines (Quanjer et al. 1993; Roca et al. 1988). The following lung function parameters were measured:

- FEV1 – forced expiratory volume in 1 s (dm^3),
- FVC – forced vital capacity (dm^3),
- FEF25-75 – forced expiratory flow rate in the range 25–75 % of FVC ($dm^3\ s^{-1}$).

The point of reference was the range of predicted values consistent with 90 % confidence interval for the population of healthy individuals studied by ECSC. All of the above-mentioned parameters were automatically calculated by spirometric software. Due to relatively small sample size (42 divers and 10 candidates for professional divers), the methods of descriptive statistical analysis were used to characterize the results.

3 Results

3.1 Forced Vital Capacity

The mean FVC was 5.6 dm^3, with a minimum at 4.3 and maximum at 7.3 dm^3. The distribution of FVC by divers' age is shown in Fig. 32.1, in which the FVC results also were compared with the 90 % confidence intervals for a sample of healthy subjects studied by ECSC. Over 95 % of the divers were more than 30 % above the predicted FVC values and 11.5 % of them

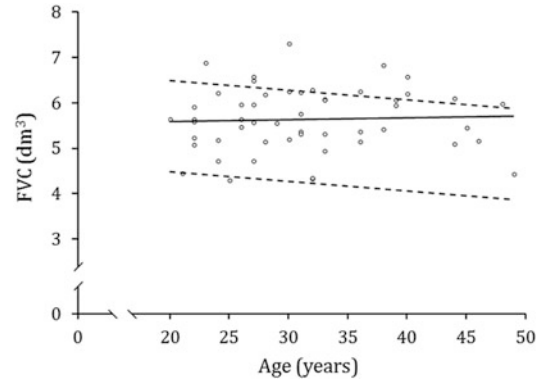

Fig. 32.1 Trend in forced vital capacity (FVC) in relation to divers' age (*solid line*) compared with 90 % confidence intervals for FVC_{max} and FVC_{min} (*dashed lines*) obtained in healthy individuals by ECSC, taken as reference

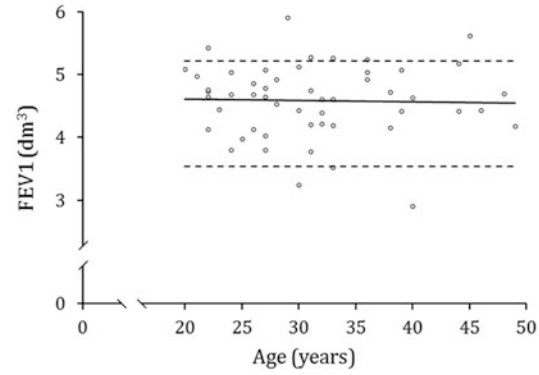

Fig. 32.2 Trend in forced expiratory volume in 1 s (FEV1) in relation to divers' age (*solid line*) compared with 90 % confidence intervals for $FEV1_{min}$ and $FEV1_{max}$ (*dashed lines*) obtained in healthy individuals by ECSC, taken as reference

(n = 6) were above the maximum FVC values with reference to the 90 % confidence interval for healthy individuals studied by ECSC.

3.2 Forced Expiratory Volume in 1 s

The mean FEV1 was 4.6 dm^3, with a minimum at 2.9 and maximum at 5.9 dm^3. The distribution of FEV1 by divers' age is shown in Fig. 32.2, in which, as was the case with FVC, the FEV1 results were compared with the 90 % confidence

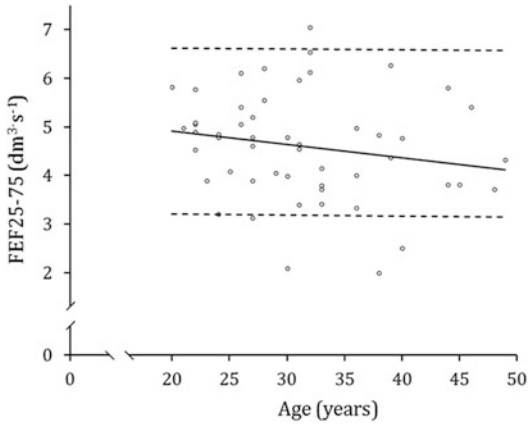

Fig. 32.3 Trend in forced vital capacity (FEF25-75) in relation to divers' age (*solid line*) compared with 90 % confidence intervals for $FEF25\text{-}75_{min}$ and $FEF25\text{-}75_{max}$ (*dashed lines*) obtained in healthy individuals by ECSC, taken as reference

intervals obtained in healthy subjects studied by ECSC. More than 70 % of the divers were in a range of 40–90 % of predicted FEV1 taken from the ECSC reference. Only did one diver not fall into the accepted 90 % confidence interval, and 10 % (n = 5) of them reached or exceeded the maximum interval values.

3.3 Forced Expiratory Flow

The mean FEF25-75 was 4.6 $dm^3\ s^{-1}$, with a minimum at 2.0 and maximum at 7.1 $dm^3\ s^{-1}$. The distribution of FEF25-75 by divers' age is shown in Fig. 32.3. The data indicate that 70 % of the divers were in a range of 50–70 % of predicted FEV1 taken from the ECSC reference. Eight percent of the divers (n = 4) did not reach and 2 % (n = 1) exceeded the accepted 90 % confidence intervals.

4 Discussion

Studies point to the phenomenon of 'large lungs' consisting of the above-average FVC values in the absence of 'air trap' markers. Lung function tests may, however, demonstrate a reduced FEV1/FVC ratio in reference to predicted values, which may indicate lung ventilation impairment of the obstructive type (Adir et al. 2005; Skogstad et al. 2000, 2002; Watt 1985; Davey et al. 1984; Crosbie et al. 1977, 1979). Deviations from the norm in spirometric tests, performed as part of a routine medical examination of divers, may be a reason to declare unfitness for work as a professional diver. However, it is not quite clear where the normal values end and abnormal begin. Some authors have pointed out that a majority of professional divers (particularly those less than 30 years old), exhibit the above-average FVC values. Still, there is a disagreement over the cause of such a phenomenon and whether a co-existing reduced FEV1/FVC ratio should be regarded as a spirometric index of pathology (Crosbie et al. 1979).

A number of studies show that spirometric indices which reflect the phenomenon of 'large lungs' in divers ought to be considered as a short-term effect this profession or a long-term negative effect of diving (Watt 1985; Davey et al. 1984; Crosbie et al. 1977, 1979). On the other hand, Bouhuys and Beck (1979) argue that the above-average FVC values in young men are observed not only among divers but also in other professional groups and in the general population. There is a belief that among individuals of the same age, similar height and weight, the major cause of this phenomenon are differences in muscle strength, which is supposed to be greater in physically trained individuals and in professionals predisposed to forced expiration training. Respiratory muscles training is the primary reason for the capability to generate increased volumes of air and increased expiratory force, which, in turn, results in the above-average FVC parameters (Clanton et al. 1987). This effect is associated with distention of alveoli and alveolar ducts, confirmed by post-mortem examination (Calder et al. 1987). Some authors suggest that the 'large lung' phenomenon is a measurable exponent of natural selection for diving rather than a sequel of diving training (Adir et al. 2005). In the present study, we found that FVC in divers was slightly increasing with age, and thus with diving experience, compared with

a natural downtrend in FVC values observed in the population of healthy individuals studied by ECSC (Fig. 32.1). The trend is consistent with the references quoted above. It should be pointed out that our results were unlikely to be distorted by the presence of candidates for professional divers among the individuals studied because all of them had extensive diving experience in recreational diving; therefore, the entire group had undergone respiratory muscles training. As regards the FEV1, the results are unambiguous. None of the studies have reported any differences due to profession done compared with the general population, e.g., divers, police officers, or submarine crews. Yet, there are certain differences in the trends developing over time (Skogstad et al. 2002; Watt 1985). A considerable reduction in FEV1 as a function of years of diving experience has been demonstrated by Tetzlaff et al. (2006) in their study of smoking divers who initially had FEV1 at a normal high level. In the present study, FEV1 in divers decreased slightly with age compared with the unchanged reference values derived from 90 % confidence interval for the population of healthy individuals studied by ECSC (Fig. 32.2). Data from the literature show a reduction the forced expiratory flow rate, particularly in the lower 25–50 % range of FVC, in divers with longer diving experience. This finding may suggest a progressive pathology of small bronchi as a function of time (Thorsen et al. 1990; Tetzlaff et al. 1988; Davey et al. 1984). Extended studies conducted in Norway in police divers beginning their careers and continued for 6 years have demonstrated a diving-related flow impairment in small airways, as compared with non-diving officers (Skogstad et al. 2000, 2002). Similar conclusions were reached by Adir et al. (2005) who examined a relatively large, but not fully representative due to specific selection criteria, group of 109 Israeli military divers. Our present findings clearly indicate that FEF25-75 in divers decreases with age (Fig. 32.3). That was a consistent tendency and it was incomparably stronger than that in case of 90 % confidence interval for the population of healthy individuals studied by ECSC.

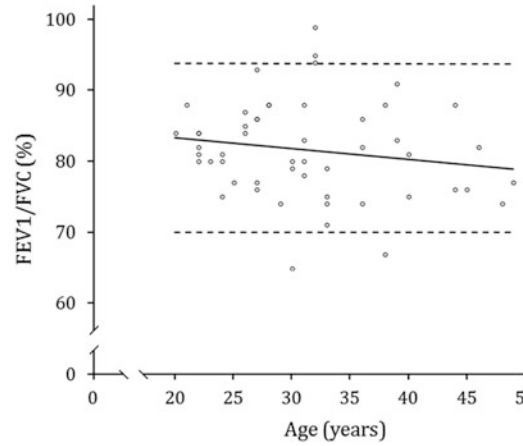

Fig. 32.4 Trend in FEV1/FVC ratio in relation to divers' age (*solid line*) compared with 90 % confidence intervals for $FEV1/FVC_{min}$ and $FEV1/FVC_{max}$ (*dashed lines*) obtained in healthy individuals by ECSC, taken as reference

The present study has limitations, stemming mostly from a single measurement technique employed. Nevertheless, the findings that support the existence of 'large lungs' in divers are consistent with the data reported by other authors. It seems that the absence of a natural downward trend in FVC in older divers, which in our study is synonymous with greater diving experience, may indicate that occupational diving leads to functional distention of a certain number of alveoli or alveolar ducts which are not normally used while breathing. However, such functional lung distention may, in the long-term, lead to ventilation impairment of the obstructive type (Thorsen 2003), a disorder which has repeatedly been observed in professional divers (Watt 1985; Davey et al. 1984; Crosbie et al. 1977, 1979). It seems that following the reduction in the FEV1/FVC ratio with time would be the best indicator of a possible progressive obstructive lung disease in professional divers (Swanney et al. 2008; Skogstad et al. 2000, 2002). In the present study, this reduction did not assume significance in older divers (Fig. 32.4). Thus, we cannot decisively state that professional divers would be prone to obstructive ventilation impairment as they continue their career until we perform longitudinal testing in the same individuals over a period of several years.

5 Conclusions

Due to a relatively small number of subjects, only were descriptive analyses of selected parameters of lung function in divers and candidates for professional divers presented in this chapter. The data are part of the medical tests which all divers must undergo before they are declared fit to dive. The present study demonstrates some differences between divers and the general population as assessed by spirometric measurements. The FVC tends to be on the higher side and FEF25-50 on the lower side in divers compared with the reference data of the general population. The results lend support for the presence of the phenomenon of 'large lungs' in professional divers. Functional hyperinflation persisting for an extended period of time may potentially lead to lung ventilation impairment of the obstructive type. Extending the observation period could demonstrate whether these trends would change after more years of diving experience. Therefore, analysis of lung ventilatory indices in divers should include the correlation of lung function as a function of years of diving experience, the nature of occupational activities carried out underwater, and the history of diseases and diving accidents.

Acknowledgments We thank Polish professional divers for their participation in this study and Ms. Alicja Klos and Dr. Piotr Siermontowski for their excellent assistance and support during collecting the data.

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

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Influence of Traffic-Related Air Pollutants on Lung Function

33

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Abstract

We investigated the influence of traffic-related air pollutants on respiratory function, with a focus on the non-smoking residents of the capital city of Warsaw in Poland, who lived close to busy streets. The results demonstrate that people living in some parts of the city show symptoms of bronchial obstruction over four times more often than those from the control group consisting of the inhabitants of a remote region in eastern Poland, with considerably less air pollution. Using multiple regression models it was shown that, apart from the place of living, the floor the apartment is situated on, the length of residence, allergy, and physical activity are the factors that significantly influence the forced expiratory volume in 1 s (FEV1) and the pseudo-Tiffenau index (FEV1/FVC).

Keywords

Bronchial obstruction • Health • Municipal environment • Pulmonary function • Traffic congestion • Traffic-related air pollutants

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_33,

1 Introduction

Studies on the influence of air pollution on human health conducted in the 1990s (Osterlee et al. 1996; Schwartz 1994; Wjst et al. 1993) point to a close connection between exposure to pollutants and the development of diseases of the respiratory, circulatory, and nervous systems. Air pollution as a factor aggravating the symptoms of chronic obstructive pulmonary disease (COPD) has been known for over 50 years, which contributed to the development of air quality standards. This has resulted in a significant fall in air pollutant emissions from combustion of fossil fuels, especially particulate matter (PM) and sulphur dioxide (Badyda et al. 2013; Klejnowski et al. 2012; Majewski and Przewozniczuk 2009). Dynamic growth of road traffic has, however, caused an increase in the level of other pollutants, such as ozone, nitrogen oxides, or particulate matter of the diameter less than 10 μm (MacNee and Donaldson 2000), especially less than 2.5 μm or even 1.0 μm. Actually, air pollution occurs in the form of a mixture of various types of gases and particular matter, the concentrations of which change depending on the measurement location, emission sources, prevailing directions of air mass movement, climatic, meteorological, and topographic conditions. The most common air pollutants are: particulate matter, tropospheric ozone, nitrogen oxides, sulphur oxides, heavy metals and aromatic hydrocarbons, especially polycyclic ones (Chen and Kan 2008; Samet and Krewski 2007; Brook et al. 2004).

As shown by Martin et al. (2010), the main air quality problem in Europe is high exposure to particulate matter and ozone, to nitrogen oxides in some regions, and to benzo(a)pyrene in case of Poland. An important factor that has a significant contribution to the current situation is road traffic, which in the Polish conditions is gaining special significance. Dynamic growth in the number of vehicles combined with much slower development of the road network, causes a noticeable reduction in average traffic velocity, which is particularly important in large urban areas. Street networks are unable to efficiently handle generated traffic. This results in increasing levels of air pollutants, which may lead to greater incidence of chronic respiratory diseases. As indicated by Keller et al. (1995), motor vehicles moving on congested streets with a low average speed significantly increase fuel consumption (over 20 dm^3 per 100 km) and as a result, emissions of air pollutants like carbon monoxide, nitrogen dioxide, polycyclic aromatic hydrocarbons, and in case of diesel engines also particulate matter.

Human health hazard is primarily conditioned by the size and chemical composition of particulate matter. Particles of the diameter below 10 μm get through the throat and nose to lower parts of the respiratory system. Those smaller than 3 μm are easily deposited in pulmonary alveoli, which may result in serious health hazard. Particles of even smaller diameters may enter to the circulatory system and move to various organs including the brain. Results of studies conducted in Germany by Franck et al. (2011) showed that exposure to solid particles of the aerodynamic diameter of 0.01 μm has an influence on the occurrence of hypertension. Most frequent consequences of long-term exposure to high levels of air pollution include chronic obstructive pulmonary disease and bronchial asthma (Andersen et al. 2011). A cohort study conducted in Denmark (Andersen et al. 2011) on a sample of over 57,000 patients showed that a year-long living close to main roads has statistically significant contribution to the increase of COPD incidence. The study proved that the hazard ratio of COPD amounts to 1.08 with an increase of interquartile range of 35-year-long average concentration of NO_2 by 5.8 $\mu g/m^3$, whereas a closer relationship was observed in patients with diabetes (1.29) and bronchial asthma (1.19). Similar study, on a Swedish example (Lindgren et al. 2009) showed that a 100-m distance of residence from a busy road (traffic intensity of over ten cars per min), in comparison with a road of small traffic intensity, was connected with the incidence of bronchial asthma (OR = 1.40, 95 % CI = 1.04–1.89) and COPD (OR = 1.64, 95 % CI = 1.11–2.4).

Nafstad et al. (2004) demonstrated that long-term exposure to air pollutants in the Norwegian

urban environment may lead to increased mortality from respiratory and cardiovascular diseases. The mortality risk factor amounts to 1.16 for respiratory diseases other than cancer, 1.11 for lung cancer, and 1.08 for ischemic coronary disease. A cohort study conducted in Canada on a sample of over 450,000 people (Gan et al. 2010) showed a linear relation between traffic-related air pollution and coronary heart disease. The incidence of heart ischemia was 1.19 times greater among residents of buildings 150m away from a motorway or over 50 m away from a main road, in comparison with people living at longer distances from busy roads. There is also a growing body of evidence that long-term exposure to air pollution is related to an increase in mortality due to cardiovascular diseases (Brunekreef et al. 2009).

An English study (Peacock et al. 2011) showed that also short-term exposure to air pollution (NO_2, O_3, SO_2, or PM_{10}) contributes to aggravation of COPD symptoms. Traveling during the rush hours, with greater vehicle traffic, and consequently with higher air pollution, may have adverse health effects. A Dutch study (Zuurbier et al. 2011) showed that even short-term exposure to high levels of particulate matter results in a decrease in respiratory system immunity and risk of inflammation. Daily exposure to high levels of pollution contributes to the occurrence of ischemic heart disease, heart failure and cardiac arrhythmia, peripheral arterial disease, and even increased risk of sudden death (Nelin et al. 2012; Autrup 2010).

In the present study we investigated the influence of traffic-related air pollutants on respiratory function, with a focus on the non-smoking residents of the capital city of Warsaw in Poland, who lived close to busy streets.

2 Methods

2.1 Subjects

The study was approved by a local Ethics Committee of the Military Institute of Medicine in Warsaw, Poland, and informed consent was obtained from all study participants.

Pulmonary function tests were conducted systematically from April to June and from September to October in the years 2008–2011. Selection of the study period was conditioned by the necessity of avoiding potential influence of short-term effects of air pollutants from sources other than traffic, e.g., municipal and domestic sources, and holiday periods, which could affect representativeness of the sample.

The analysis encompassed 4,725 results of pulmonary function tests. In Warsaw, 3,834 people living in the vicinity of 7 selected busy roads were tested, including 1,608 women and 2,226 men aged 9–91 (mean age 51 $\pm$ 20 years). The proportion of non-smokers was 50.5 % (1,938 people). The control group consisted of 891 individuals living in rural areas isolated from direct impact of traffic-related air pollutants emission. The group included 471 women and 420 men aged 9–91 (mean age 50 $\pm$ 20). 50.4 % of the group (449 people) were smokers. The results of the tests from patients presently treated for chronic obstructive pulmonary disease (COPD) or bronchial asthma, as well as those who did not cooperate with the examiner were excluded from further analysis.

2.2 Protocol

The examination was conducted according to the following scheme:

- information about the aim of the examination and the lack of its harmful impact on the human body;
- subjective research – a questionnaire including: information on place of residence (locality, floor, distance to main roads, period of residence, etc.) and its conditions (heating method, gas cookers, other air pollutants emitters), smoking habit and its intensity, passive smoking, exposure to harmful factors in workplace and living place, current pulmonary diseases, presence of respiratory disease symptoms, allergies and anthropometric data;
- objective research – pulmonary function test carried out in the sitting position (Easy One spirometers; AeroMedika, Warsaw, Poland) after a few-minute time given to adapt to the

new breathing conditions. Several flow-volume curves were recorded, until repeatability in accordance with the American Thoracic Society (ATS) criteria was achieved. The test results included following parameters:

- FVC (forced vital capacity);
- FEV_1 (forced expiratory volume during the first second of expiration);
- FEF_{50} (forced expiratory flow at 50 % of FVC);
- FEV_1/FVC (the so-called pseudo-Tiffeneau factor);
- PEF (peak expiratory flow).

According to the ATS and the Polish Respiratory Society guidelines, research was carried out until at least three repeatable results were obtained, i.e., results for which the values of indicators for particular measurements did not vary by more than 5 %. Predicted values were calculated according to commonly used ERS/ECCS standards (Quanjer et al. 1993).

The Shapiro-Wilk test was used to assess normality of data distribution. Data were compared using both parametric, ANOVA, and non-parametric, Kruskal-Wallis, tests as required. A $p < 0.05$ was accepted as a statistically significant level. Multiple regression models were used to assess factors determining variability of spirometric indices. Statistical analysis was conducted using Statistica 9.1 software.

3 Results and Discussion

Presentation of the results is limited to the non-smoking persons only in order not to confuse cigarette and traffic-related air pollutants. The non-smoking group included 2,387 inhabitants, including 809 women and 1,578 men, aged 9–91 (mean age 54 ± 21 years), comprising 1,938 Warsaw residents and 449 rural areas inhabitants. Both city and rural areas inhabitants had spirometric indices within the accepted norms. However, there were significant differences between the two groups. The mean values of FEV_1, FEF_{50}, and the FEV_1/FVC ratio were lower in the city residents ($p < 0.05$). Moreover, significant differences were also noted in case of PEF. Overall, the results indicate the adverse effects on lung function of living in a big city compared with rural areas, excluding the potential confounder of cigarette smoking (Table 33.1).

We also calculated the percentages of people with symptoms of bronchial obstruction. The symptoms were broken down by the degree of obstruction severity, where bronchial obstruction was diagnosed when the FEV_1/FVC values were below 70 %, and mild obstruction was assumed for $FEV_1 \geq 80$ %, moderate for FEV_1 50–79 %, and severe for $FEV_1 \leq 50$ %predicted (Table 33.2). In all categories of obstruction above outlined the percentage of afflicted individuals was several-fold higher in Warsaw City than rural areas inhabitants.

To sum it up, the results of this study demonstrate the following:

- decreases in the essential spirometric indices regarding the assessment of bronchial patency in the big city residents compared with the inhabitants of rural areas with no appreciable traffic-related air pollution;
- 4.1-times more people with bronchial obstruction among the Warsaw residents compared with those living in rural areas;

Table 33.1 Spirometric indices in non-smoking inhabitants of Warsaw and rural areas

Spirometric variable	Warsaw group	Rural area group
FEV_1	95.3 ± 19.3**	100.3 ± 17.2
FVC	107.5 ± 24.9	108.1 ± 18.9
PEF	96.2 ± 24.2*	100.5 ± 22.8
FEF_{50}	75.0 ± 32.8**	86.0 ± 31.3
FEV_1/FVC	94.5 ± 14.4**	98.6 ± 10.6

Data are mean %predicted values ± SD

*$p < 0.05$; **$p < 0.001$ for the differences between the two groups (Kruskal-Wallis test)

Table 33.2 Percentages of non-smokers with airflow obstruction in urban and rural areas groups

Obstruction severity	Warsaw group	Control group
Mild	3.1	1.1
Moderate	4.4	0.7
Severe	1.7	0.5
All	9.1	2.2

- rather a moderate degree of bronchial obstruction among the city residents, with the FEV_1 level in a 50–79 % predicted range. This level of obstruction was found in the present study in the non-smoking individuals residing in a big city with traffic-related air pollution. A similar level of obstruction is routinely found in smokers suffering from COPD, and the existence of obstruction is not often appreciated in people exposed mainly to environmental factors.

The etiology of chronic pulmonary diseases includes mainly smoking and air pollution. Therefore, it may be assumed that among non-smokers, low values of sensitive lung function variables and over a four-times higher percentage of people with obstruction in the group of city inhabitants living close to streets with heavy traffic, as opposed to those living in unpolluted rural areas, could be a result of exposure to excessive concentrations of pollutants. We further performed the assessment of factors which could influence spirometric variables using multiple regression models. For this purpose, the relationship described in Eq. 33.1 below was used:

$$\hat{Y} = b_0 + b_1X_1 + b_2X_2 + \ldots + b_kX_k \quad (33.1)$$

where:

$\hat{Y}$ – predicted value of the dependent variable Y;
$X_1, X_2, \ldots, X_k$ – independent variables;
$b_1, b_2, \ldots, b_k$ – regression coefficients

Regression coefficients were estimated using the maximum likelihood method. Separate models for FEV_1 and FEV_1/FVC were created. The models contain only the independent variables that significantly ($p < 0.05$) influence the variability of dependent variables. The models for FEV_1 and FEV_1/FVC are given in Eqs. 33.2 and 33.3, respectively:

$$\begin{aligned} FEV_1\% = & 108.795 - 6.942 \cdot LIV \\ & - 0.092 \cdot TLV + 0.329 \cdot FLR \\ & - 3.198 \cdot ALL + 3.974 \cdot SPR \\ & - 0.088 \cdot AGE - 2.086 \cdot GEN \end{aligned} \quad (33.2)$$

$$\begin{aligned} \frac{FEV_1}{FVC\%} = & 103.701 - 4.729 \cdot LIV \\ & + 0.323 \cdot FLR + 1.289 \cdot SPR \\ & - 0.110 \cdot AGE \end{aligned} \quad (33.3)$$

where:

LIV–place of living – dichotomous variable; rural area inhabitants (LIV = 0), urban area inhabitants (LIV = 1); TLV–period of living (years); FLR–floor of residence (0,1,. . .,n); ALL–allergies – dichotomous variable; no allergies (ALL = 0), presence of allergies (ALL = 1); SPR–sports activities – dichotomous variable; no physical activity (SPR = 0), presence of physical activity (SPR = 1); AGE–age of investigated person (years); and GEN–gender – dichotomous variable; man (GEN = 0), woman (GEN = 1).

The elaboration of the models demonstrates that the predicted values of both FEV_1 and FEV_1/FVC decline:

- among city inhabitants;
- with increasing period of living in a particular place, which is partially related with age;
- with decreasing floor of residence;
- with the presence of allergy;
- among sedentary people not practicing physical activity;
- with increasing age of a person;
- in female gender.

The decline appeared statistically more expressed for FEV_1 than that for FEV_1/FVC.

4 Conclusions

Among the non-smoking people living in the vicinity of busy roads in a large city, symptoms of bronchial obstruction are over four times more frequent than in rural areas inhabitants. The essential spirometric variables FEV_1, FEV_1/FVC, and FEF_{50} were appreciably lower in the city than rural inhabitants. This points to an increased percentage of people who exhibit inflammatory reactions in the respiratory system due to exposure to air pollution. The observation was confirmed by the results of multiple regression models pointing to the number of factors that worsen the spirometric variables, particularly in the city inhabitants. The lower floor of residence in buildings, the extended time of living close to busy streets, advancing age, lack of physical activity, and allergy all have a negative influence on lung function. Thus, heavy traffic-related air pollution is an essential factor impairing health in big cities.

Acknowledgements This work was financed by the Polish budget for science for 2008–2012.

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

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Fractional Exhaled Nitric Oxide in Clinical Trials: An Overview

34

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Abstract

Designing clinical trials in asthma it is crucial to find the perfect primary endpoint for showing bioequivalence, especially when the investigational medicinal product is not a bronchodilator, but a substance, which suppresses the inflammatory process, e.g. inhalative corticosteroids (ICS). In the past, lung function parameters were used as the primary endpoint, which entails a long study duration and hundreds of patients. The measurement of fractional exhaled nitric oxide (FeNO) is established as a non-invasive marker for eosinophilic inflammation, and several guidelines focus on that diagnosis. FeNO is a surrogate measure of eosinophilic inflammation and at the same time, eosinophilic airway inflammation is usually steroid responsive. Thus, FeNO should be a part of the clinical management of asthma in ambulatory settings in conjunction with other conventional methods of asthma assessment. Furthermore, FeNO should be used to determine the presence or absence of eosinophilic airway inflammation, to determine the likelihood of steroid responsiveness, to measure response to steroid therapy, and level of inflammation control. In addition, FeNO is a useful tool to monitor patient ICS treatment adherence and allergen exposure. FeNO may be used to predict steroid responsiveness and as a measure to determine the optimal treatment of airway inflammation. FeNO has all characteristics of a good marker for bioequivalence measurements in the market approval process of generic ICS products. With a reliable study design in terms of patient population, concomitant medication,

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_34,

equipment and other factors, which can influence the measurement, efficient clinical trials can be performed, with a relatively short treatment time of 2–4 weeks and 50–100 patients.

Keywords
Asthma control • Bioequivalence • Clinical trial • Fractional exhaled nitric oxide • Inhaled corticosteroids • Pharmacodynamics

1 Introduction

Asthma is a common clinical syndrome that involves multiple disease processes. It is highly variable in its underlying pathology, clinical manifestations, natural history, and response to treatment (Hakonarson and Halapi 2002). The worldwide prevalence of asthma is increasing, especially in the industrialized countries (GINA Asthma Management and Prevention 2011), where it has become the most common chronic disease among children. One of the changes in asthma management over the last decades is the movement from medication that relieves symptoms and treats the underlying inflammation in the airways to real asthma management, which involves closer care to the patients (Garbutt et al. 2009; Holgate et al. 2008; Bender et al. 2008).

New approaches in asthma diagnosis are implemented. There is a search for new tools and parameters to distinguish phenotypes of patients, e.g., who would or would not respond to inhaled corticosteroids (ICS) (Redington et al. 2001). The measurement of fractional exhaled nitric oxide (FeNO) is such a tool and is established as a non-invasive marker for eosinophilic inflammation. Several guidelines focus on that diagnosis tool and describe the method and its appliance in detail. The American Thoracic Society's (ATS) guidelines for FeNO measurement are the basis of the following introduction (ATS/ERS Recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide 2005; ATS/ERS Recommendations for Standardized Procedures for the On-line and Off-line Measurement of Exhaled Lower Respiratory Nitric Oxide and Nasal Nitric Oxide in Adults and Children 1999).

Diagnosis of asthma can be difficult due to the number of different diseases with similar symptoms. Therefore, there is no single measure or instrument which can provide definitive proof that asthma is present. Amongst physical examination, asthma symptoms and lung function testing, FeNO plays an increasingly important role as a marker of airway inflammation. Levels of FeNO are elevated in patients with asthma who are not taking ICS, compared to healthy subjects (GINA, Asthma Management and Prevention 2011). FeNO increases within 10 min in asthmatic patients, when they are challenged with silver (argentum, Ag), whereas healthy control individuals have no change in FeNO (Dweik et al. 2001).

FeNO is a surrogate measure of eosinophilic inflammation (Taylor et al. 2006) and at the same time, eosinophilic airway inflammation is usually steroid responsive (Meijer et al. 2002; Pizzichini et al. 1999). It originates mostly from the epithelium, by inducible nitric oxide (NO) synthase that is sensitive to steroids (Redington et al. 2001). Therefore, FeNO may be used to predict steroid responsiveness (Hahn et al. 2007; Smith et al. 2005a, b) and as a measure to determine the optimal treatment of airway inflammation (Shaw et al. 2007; Smith et al. 2005a, b).

National Jewish Health published a consensus statement on the clinical use of FeNO measurement (National Jewish Health, Consensus Statement on the use of Fractional Exhaled Nitric Oxide (FeNO) in the Clinical Management of Asthma 2009). The consensus states that FeNO

should be a part of the clinical management of asthma in ambulatory settings in conjunction with other conventional methods of asthma assessment. Furthermore, FeNO should be used to determine the presence or absence of eosinophilic airway inflammation, to determine the likelihood of steroid responsiveness, to measure response to steroid therapy, and level of inflammation control. In addition, FeNO is a useful tool to monitor patient ICS treatment adherence and allergen exposure.

1.1 Statements on the Use of FeNO as a Marker in Clinical Trials

Generally, several studies showed the response of FeNO to ICS of different kind. A German a study showed that FeNO decreased from 14.8 ± 1.9 ppb to 7.7 ± 1.2 ppb within a 4-week treatment period in 31 children with mild-to-moderate asthma. Patients inhaled budesonide from Turbuhaler 200 μg twice daily. FeNO rose back to the original value of 14.0 ± 1.2 ppb after 4 weeks of wash-out (no therapy). A positive correlation between the patients' compliance in taking their prescribed inhaled steroid medication and the reduction of FeNO was established (Beck-Ripp et al. 2002).

An U.S. dose-response study of FeNO after ICS included 15 adult asthmatic patients with baseline FeNO >60 ppb. After the inhalation of budesonide twice daily from a metered-dose inhaler (MDI) for 1 week, FeNO decreased from 103.5 ppb (78.5–136.7 ppb) to 37.4 ppb (29.1–48.0 ppb) regardless of the budesonide dose of 100 μg/d, 400 μg/d, or 800 μg/d (Silkoff et al. 2001).

Currie et al. (2003) performed a study with 25 moderate adult asthmatic patients after a 4 weeks steroid wash-out, who inhaled either salmeterol (50 μg) alone or a fluticasone/salmeterol (100 μg/50 μg) combination. After a 2-week treatment of one puff twice daily, the effect of salmeterol/fluticasone compared with salmeterol was 4.3/1.3-fold for sputum/blood eosinophils, 1.8-fold for FeNO (Currie et al. 2003).

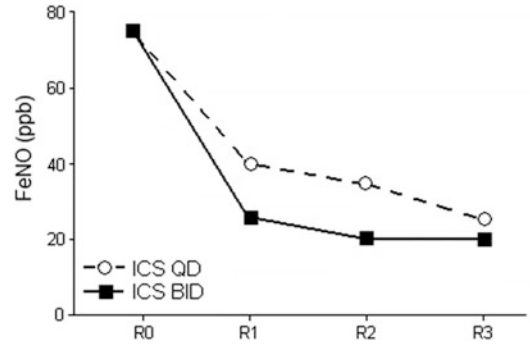

Fig. 34.1 Example of the dose response of three different dose strengths (R0 = no dose, R1, R2, R3) inhaled either once daily (QD) or twice daily (BID). (Freely adapted from Kim et al. 2012)

1.2 Statements on the Use of FeNO as Marker in Clinical Trials to Show Bioequivalence

Chowdhury et al. (2010) from FDA stated in his presentation at Respiratory Drug Delivery (RDD) conference in 2010 that FeNO is a good marker for bioequivalence measurements in the market approval process of generic ICS products. According to the draft guidance document of the Canadian Health Authorities, the use of FeNO as an alternative biomarker may be considered if adequate justification is provided and the study design is considered acceptable (Health Canada, Draft Guidance Document 2011).

1.3 Expected Dose-Response Relationship of ICS and FeNO

Since the dose response relationship is not yet established for most of the ICS, Kim et al. (2012) provided suggestions for the study design, beginning with a pilot study to establish the response in FeNO to the respective ICS in its different dose strength (Fig. 34.1) The next step is a pivotal trial, in which bioequivalence will be shown.

2 Methods

2.1 Equipment for the Measurement of FeNO

Silkoff et al. (2004) described an Aerocrine device, NIOX, which measures FeNO according to the guidelines. This device has been approved by the FDA and fulfills the criteria of the FDA guidance for industry in 2003 (ATS/ERS Recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide 2005; FDA Guidance for Industry and FDA Staff 2003).

NIOX MINO is the device of choice globally used for measuring airway inflammation in clinical practice and in clinical studies. The performance of NIOX MINO is documented in more than 30 clinical papers in peer-reviewed journals (Dweik et al. 2011). It is relatively easy to use, maintenance free and its values are quality assured.

2.2 Measurement of FeNO

There is a consensus that FeNO is best measured before spirometric maneuvers, at an exhaled rate of 0.05 L/s, maintained within 10 % for more than 6 s, and with an oral pressure of 5–20 cmH_2O to ensure velum closure. Results are expressed as the NO concentration in ppb based on the mean of two or three values within 10 % (ATS/ERS Recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide 2005; Kharitonov and Barnes 2003; Kharitonov et al. 1997; Bratton et al. 1999).

Normal ranges were published for adults by Kharitonov et al. (1997) and Olin et al. (2007). A reasonable estimate of the normal range for FeNO in healthy adults is less than 35 ppb (Kharitonov and Barnes 2003; Buchvald et al. 2005). In corticosteroid naïve patients with non-specific respiratory symptoms, high FeNO levels (>50 ppb) are associated with a significant clinical response to ICS independent of the final diagnosis (Smith et al. 2005a, b). FDA recommends the online measurement of FeNO at the ATS recommended flow rate of 0.05 L/s.

2.3 Factors Affecting FeNO Levels

According to the FDA recommendation, factors which could influence the measurement should be taken into account (FDA Guidance for Industry and FDA Staff 2003) (Table 34.1). The same is recommended by the guidance for industry FDA 1995 (FDA Guidance for Industry and FDA Staff 1995).

2.4 Diseases Affecting FeNO Levels

Diseases affecting exhaled NO values are listed in Table 34.2.

3 General Considerations of Designing a Clinical Trial with FeNO as a Primary Endpoint, with Emphasis on Bioequivalence

3.1 Primary and Secondary Efficacy Endpoints

Exhaled NO is considered a biologically relevant marker of airway inflammation because it is shown to increase in persistent asthma due to airway inflammation (Taylor et al. 2006) and it appears to provide a dose-response effect (Kim et al. 2012).

3.2 Secondary Efficacy Endpoint

FEV_1 is recommended as a secondary clinical endpoint because it is influenced secondarily by the drug effect on inflammation and by other factors. Other secondary endpoints (such as asthma symptoms or Asthma Control Questionnaire) may be considered as additional to FEV_1 (Health Canada, Draft Guidance Document 2011).

Table 34.1 Non-disease-related patient factors influencing exhaled NO values

Influence on FeNO	Factor/recommendation
Sex, age, height	Sex and age are major factors which influence exhaled NO measurements. (Taylor et al. 2007; Olin et al. 2006; Buchvald et al. 2005; Avital et al. 2003)
Spirometric maneuvers	Spirometric maneuvers transiently reduce exhaled NO levels (Silkoff et al. 1999). Recommendation: perform NO analysis before spirometry (ATS/ERS 2005)
Airway caliber	FeNO levels vary with the degree of airway obstructions or after bronchodilation (Kissoon et al. 2002; Silkoff et al. 1999; Silkoff et al. 1998; de Gouw et al. 1998; Yates et al. 1997). Recommendation: perform NO analysis always after the same time after bronchodilation
Food/beverages	FeNO levels increase after the ingestion of nitrate-rich diet. (ATS/ERS 2005; Olin et al. 2001; Zetterquist et al. 1999) Recommendation: patients should refrain from eating at least 1 h before NO analysis (ATS/ERS 2005)
Caffeine	Influence on FeNO levels not yet been clarified. Recommendation: patients should refrain from caffeine, since it might influence spirometry
Circadian rhythm	There is inconsistency in the results reported in the literature about the influence of circadian rhythm. Recommendation: to measure FeNO at the same period of the day and to record the time of measurement (ATS/ERS 2005)
Exercise	Exercise reduces FeNO significantly (Evjenth et al. 2012). Recommendation: patients should avoid exercise for 1 h before the measurement (ATS/ERS)
Smoking	Peak FeNO is significantly reduced in smokers, with a strong relation between FeNO and cigarette consumption (Kharitonov et al. 1995; Persson et al. 1994). Passive smoking causes only a transient (*ca* 30 min) reduction of FeNO (Maniscalo et al. 2002; Yates et al. 2001). Recommendation: only non-smokers or ex-smokers (6 months) may be enrolled. Patients have to refrain from smoking throughout the study
Medications	ICS and orally taken corticosteroids have an influence on FeNO; therefore, the only corticosteroid to be taken is the study medication. NO synthase inhibitors (Yates et al. 1996) and leukotriene-axis modifiers (Bratton et al. 1999; Bisgaard et al. 1999) reduce FeNO and should be excluded. NO donor drugs (Marczin et al. 1997) and L-arginine increase FeNO (Mehta et al. 1996; Kharitonov et al. 1995) and should be excluded

Table 34.2 Diseases affecting exhaled NO values

Airway infection	Upper and lower respiratory tract viral infections may lead to increased levels of exhaled NO in asthma (de Gouw et al. 1998; Kharitonov et al. 1995). Recommendation: FeNO measurements should be deferred until recovery, if possible, or the infection should be recorded in the chart. HIV infection is associated with a reduction in exhaled NO (Loveless et al. 1997)
Atopy, allergic rhinitis, IgE levels	Levels of FeNO and immunoglobulin E (IgE) tend to be increased when atopy or allergic rhinitis are present (Olin et al. 2006; van Amsterdam et al. 2003; Jouaville et al. 2003). Beta-2-mimetics do not seem to influence the NO measurements; therefore, patients do not need to refrain from inhaled beta-2-mimentics (Currie et al. 2003)
Pulmonary hypertension	Reduced FeNO (Rolla et al. 2000)
Cystic fibrosis	Reduced FeNO (Narang et al. 2002)
Ciliary dyskinesia	Reduced FeNO levels (Narang et al. 2002)

Adapted from Taylor et al. (2006)

3.3 Study Population

For ICS, asthmatics with high levels of exhaled NO appear to offer a better chance for establishing a dose-response relationship (Silkoff et al. 2001). Since asthma patients with an eosinophilic inflammation are more likely to react to ICS and therefore show an improvement in FeNO, the inclusion criterion should be asthma with an increased FeNO level of at least 45 ppb

(Chowdhury et al. 2010). The ATS guidelines (Dweik et al. 2011) recommend that a FeNO greater than 50 ppb can be used to indicate that eosinophilic inflammation and the responsiveness to ICS is likely. In terms of severity, patients with mild to moderate asthma should be enrolled, since those patients are more likely not to have need for continuous ICS and can therefore be included in an off-treatment-phase. Patients should be stable and chronic.

3.4 Sample Size Estimation

Sample size of the study should be calculated based on the primary efficacy endpoint, the FeNO, to ensure there is a reasonably powered sample size to demonstrate therapeutic equivalence. A pilot study is used to provide information on the mean and variance of the nonlinear pharmakodynamic response and is used to calculate the sample size for a pivotal trial. To address this nonlinearity, a dose scale approach on an E_{max} model can be utilized, such that equivalence of local delivery is established based on the ratio of the test and reference drug product's delivered dose (Lee 2012; Kim et al. 2012).

3.5 Treatment Interval

The time span of response to ICS has not been adequately addressed; estimates for the time span range from 3 days (Kharitonov et al. 2002) to 8 weeks (Massaro et al. 1995). Since other studies found an effect after 1 week (Silkoff et al. 2001), after 2 weeks (Currie et al. 2003) or after 4 weeks (Beck-Ripp et al. 2002), it seems to be the most reasonable to apply a treatment interval of 2–4 weeks. This should allow sufficient time to see a clinically significant inflammatory improvement in the patients.

4 Clinical Study Design to Show Bioequivalence

Generally, FDA suggests for the bioequivalence tests of corticosteroids two in vivo studies, a pilot dose-duration response study with the reference product only, and a pivotal in vivo bioequivalence study, comparing test and reference product (FDA Guidance for Industry and FDA Staff 1995).

4.1 Pilot Study

The relationship between dose (D_R) and the observed response (E_R) of the reference product has to be established in the pilot study, measuring FeNO. The pilot study will be performed with three different doses of the reference product (R1, R2, R3) administered once daily in a randomized order (Kim et al. 2012). If a crossover study design will be chosen, a washout period (WA) should eliminate the carry-over effects (see Fig. 34.2) (Health Canada, Draft Guidance Document 2011). The primary efficacy endpoint, the FeNO, will be measured at baseline and at the last day of each treatment. The FeNO data from the pilot study will provide information about mean and variance of pharmacodynamic response on each dose as well as interrelationships amongst doses (Kim et al. 2012).

4.2 Pivotal Study

The pivotal study will be performed with the three doses of reference product (R1, R2, R3) and the lowest dose of the test product (T1), administered once daily in a randomized order. As described in the pilot study, a washout period will be performed to eliminate carry-over effects (Health Canada, Draft Guidance b Document 2011).

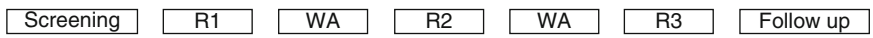

Fig. 34.2 Pilot study design (*R1*, *R2*, *R3*, Reference product; *WA*, washout phase)

5 Conclusion

The measurement of FeNO concentration in exhaled breath is a quantitative, noninvasive, simple, and safe method of measuring airway inflammation that provides a complementary tool to other ways of assessing airway disease, including asthma. A clinical practice guideline has been designed to guide clinicians how exhaled NO measurements should be used and interpreted (Dweik et al. 2011; ATS/ERS Recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide 2005).

The FDA published a guidance document for the Breath Nitric Oxide Test System 26. It suggests in vivo studies for the bioequivalence tests of corticosteroids; a pilot dose-duration response study with the reference product only and a pivotal bioequivalence study that compares test and reference products (FDA Guidance for Industry and FDA Staff 1995). National Jewish Health published a consensus statement on the clinical use of FeNO measurement (National Jewish Health Consensus statement on the use of fractional exhaled nitric oxide (FeNO) in the clinical management of asthma 2009). According to the draft guidance document of the Canadian Health Authorities, the use of FeNO as alternative biomarker may be considered if adequate justification is provided and the study design is considered acceptable (Health Canada, Draft Guidance Document 2011).

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

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Real-Time Breath Analysis in Type 2 Diabetes Patients During Cognitive Effort

35

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Abstract

The understanding the functional expression of exhaled volatile organic compounds (VOCs) has gradually expanded from the initial identification of breath pathological markers to direct expression of physiological activity. In the present study we investigated the potential application of breath analysis in real-time monitoring of type 2 diabetes mellitus (T2DM) patients *versus* control subjects while performing a cognitive task. T2DM is associated with cognitive impairment and neural deficits, because of insulin resistance and high expression of insulin receptors in the hippocampus. We set out to seek the evidence for mutual associations among breath exhale, on the one side, and T2DM and cognitive effort, on the other side. The recording system consisted of a metal oxide semiconductor (MOS) which is able to detect a broad range of volatile organic compounds. The sensor provides a measure of VOCs as ppm CO_2 equivalents. The MOS is suitable for a non-invasive real-time monitoring of the breath exhale in humans. The study demonstrates that, apart from the T2DM metabolic derangement, performing a cognitive task, taken as

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DOI 10.1007/978-94-007-6627-3_35,

an index of central neural effort, evoked distinct alterations in exhaled breath content. We conclude that exhaled breath content measurement might offer a novel diagnostic and therapeutic non-invasive approach in metabolic and neurodegenerative derangements.

Keywords

Breath analysis • Continuous monitoring • Diabetes type 2 • Metal oxide semiconductor sensor • Cognitive effort

1 Introduction

Breath analysis has a potential to replace blood and urine analyses for medical investigations in several diseases. Being non-invasive, breath analysis is a particularly suitable method for neonates, children, and elderly patients (Mazzatenta et al. 2013a); it also is helpful in subjects afflicted with neurodegenerative disease like Alzheimer's. Modern breath analyzers, based on new generation of sensors, are suitable for a real-time investigation (Mazzatenta et al. 2013b). Furthermore, the traditional invasive clinical diagnostic tests are based on analyses that are usually concerned with the large molecular weight non-volatile compounds such as proteins and ions. It is now known that volatile compounds (VOCs), which reflect normal and pathophysiological products of metabolism of the body, are present in the blood. VOCs can cross the alveolar interface and are released in the exhaled air. These volatile compounds can be valuable indicators of metabolic status, enabling the distinction between healthy and diseased states, their amount can be measured with an acceptable accuracy at ppm, ppb, or lower levels (Solga and Risby 2010). An average breath sample contains about 200 VOCs out of the 3,450 different VOCs found in humans. Common VOCs in the exhaled breath of healthy subjects are isoprene (range 12–580 ppb), acetone (range 1–1,880 ppb), ethanol (range 13–1,000 ppb), methanol (range 160–2,000 ppb), and other alcohols (Solga and Risby 2010). Elevated levels of these metabolites are known to be indicative of pathological conditions; renal impairment raises breath isoprene and ammonia, and acetone is closely related to diabetes mellitus (Solga and Risby 2010).

Type 2 diabetes mellitus (T2DM) is typically a chronic metabolic disorder that is characterized by high blood glucose in the context of insulin resistance and relative insulin deficiency (Vijan 2010). This is in contrast to type 1 diabetes mellitus in which there is an absolute insulin deficiency due to destruction of the Langherans islet cells in the pancreas. The etiology is up to about 90 % of cases of T2DM, with the remaining 10 % due primarily to type 1 and gestational diabetes. Interestingly, one of the classic symptoms of T2DM is cognitive dysfunction and increased risk of dementia (Biessels et al. 2008). Diabetes, particularly in older subjects, increases the risk in the following areas of cognitive function: cognitive decline and its rate, and the risk of future dementia (Williamson et al. 2012; Cukierman et al. 2005). T2DM also is strongly related with the dementia of Alzheimer's disease. Cognitive deficits observed as a sequel of a T2DM are due in large part to impaired central insulin modulation of cognitive and metabolic processes in the hippocampus and beyond, where insulin signaling and insulin receptors are critical components of memory and cognitive processing. In a study on training in spatial memory tasks, McNay and Recknagel (2011) have demonstrated increased expression of hippocampal insulin receptors. Intrahippocampal delivery of physiological insulin doses acutely improved

performance in the hippocampal-dependent spatial working memory tasks. Importantly, these authors also demonstrated that specific blockade of intrahippocampal insulin produces a very large impairment of memory processing; the impairment was of the magnitude as that produced by, e.g., intrahippocampal administration of morphine. Under identical conditions, it has also been shown that diet-induced insulin resistance, a rodent model of T2DM, impairs both memory performance and attenuates the augmentation of that performance by intrahippocampal insulin, as well as reduces hippocampal activity of the phosphatidylinositol-3-kinase (PI3K) signaling pathway, consistent with previous findings in obese rats. Taken together these data make it clear that insulin plays a critical role in hippocampal memory processing and that systemic insulin resistance, such as that seen in T2DM, is accompanied by hippocampal insulin resistance (McNay et al. 2010).

It is now widely accepted that insulin plays an important role in the central nervous system (CNS). The circulating insulin, via an active receptor-mediated transport system, crosses the blood–brain barrier and insulin receptors are widely expressed in the brain, mainly in the hypothalamus, the hippocampus, and the cerebral cortex. Insulin with other peptides, like ghrelin or cholecystokinin, is part of the complex signaling network in the hypothalamus that regulates anabolic and catabolic balance (Ketterer et al. 2011). The brain target areas of insulin are, in particular, the hippocampus and the hypothalamus; both being involved in cognition and body homeostasis regulation. In the present study we investigated the hypothesis that performing a cognitive task, taken as an index of central neural fatigue, might have a reflection in alterations of exhaled breath content and that reflection might be different in the neuropathy-prone disease process, exemplified by T2DM. Therefore exhaled breath content measurement might offer a novel diagnostic and therapeutic non-invasive approach in metabolic and neurodegenerative derangements.

2 Methods

The procedures were performed in accord with the Ethical Standards of the Helsinki Declaration of the World Medical Association and were accepted by a local Ethics Committee. Three T2DM patients (mean age of 66 ± 5SD years), with adult-onset, treated with variable success by oral metformin diabetes, and three healthy control subjects (mean age of 63 ± 7years), without any chronic or and neurodegenerative pathologies, were enrolled into the study. The duration of diabetes in the patients was 10, 13, and 14 years from the time of diagnosis, and the diabetes-related indices at the time of this study were as follows: HgbA1c 12.9, 9.5, and 7.1 g/dl; glucose in serum 500, 253, and 200 mg/dl. All subjects provided written consent to participate in the study after being informed about the goal of the study. The VOCs content of exhaled breath was measured in all subjects in the control condition and while performing a cognitive task consisting of solving Sudoku puzzles (Table 35.1).

The recording system consisted of an iAQ-2000 (Applied Sensor, Warren, NJ) equipped with a metal oxide semiconductor (MOS), which is able to detect a broad range of volatile organic compounds (for details of the method see Mazzatenta et al. 2013b). The sensor provides a measure of VOCs as ppm CO_2 equivalents, indicated as VOCe.

The experiment was performed in a room with a controlled temperature and balanced O_2/CO_2 concentration. The protocol consisted of an initial 5 min adaptation recording to enable the subject to relax and adapt to the experimental

Table 35.1 Sudoku puzzles used in cognitive tasks

a

4	2		
		2	4
3	1		
		1	3

b

		3	4
3	4		
1	2		
		1	2

c

1		4	
	4		3
3	1		
		3	1

environment, followed by three experimental phases of 30 s each, termed pre-test, test, and post-test. The adaptation phase was used to establish the normal breath signal. The pre-test signal was the normal free breathing signal acquired after the adaptation phase and it was used for comparison with the signal recorded during the test phase. The test phase was a breathing signal recorded during cognitive effort, and the post-test was the recovery breathing signal after the cognitive effort.

The breath signal was subject to baseline correction. The total amount of VOCe was calculated as an integral of the signal curve in a time interval. The normalization applied was according to the following formula:

$$X = \left(x_i - x_{i,j}\,\mathrm{min}\right) / \left(x_{i,j}\,\mathrm{max} - x_{i,j}\,\mathrm{min}\right)$$

where X is the normalized value obtained from the result of subtraction from a value x the minimal value in a series of values ranging from *i* to *j* divided by the result of subtraction from the maximal value in the same series the minimal value. An example of the normalization formula in a series of values of 54, 23, and 68 is this: (54–23)/(68–23) = 31/45 = 0.69; the 23 becomes 0; and 68 becomes 1.

Data are given as means ± SD. Statistical elaboration consisted of using a two-tailed unpaired or paired *t*-test, as required, and one-way ANOVA followed by Dunnett's multiple comparisons *post hoc* test. A $p < 0.05$ was taken as indicative of statistically significant differences for all comparisons.

3 Results

The control subjects breathed approximately 10 ± 2 times per min, with individual breaths that had tidal volume of about 0.6 ± 1.5 l. The diabetic subjects breathed 12 ± 2 times per min, with individual breaths of 0.55 ± 1.2 l. A total amount of VOCe in the 30-s pre-test phase was appreciably higher in the healthy control than diabetic subjects; the mean values of $5.36e^5 \pm 3.19e^4$ vs. $4.32e^4 \pm 5.15e^3$, respectively; $p < 0.0001$ (unpaired *t*-test) (Fig. 35.1)

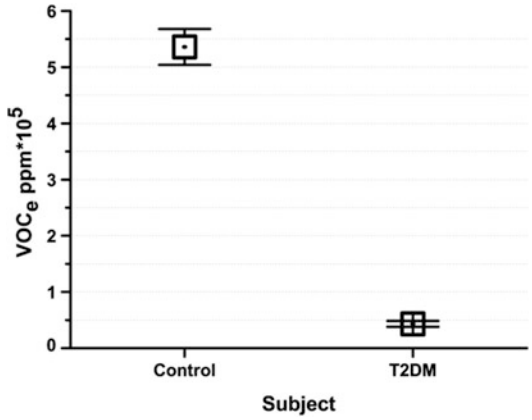

Fig. 35.1 **Comparison of the averaged VOCe**, which is a measure of VOCs as ppm CO_2 equivalents provided by the MOS sensor, in control vs. diabetic (T2DM) subjects. Measurements are from the 30-s pre-test interval

Figure 35.2 shows a normalized breath signal in a representative healthy control as compared with that in a T2DM subject throughout the experimental phases, i.e., pre-test, test, and post-test. The effects on the breath signal of the cognitive task were verified with one-way ANOVA in both control and T2DM subjects. The mean breath signal was significantly different among the experimental phases in the control subjects ($F(2,4) = 13.74$, $p < 0.01$): mean pre-test = $5.36e^5 \pm 5.53e^3$, mean test = $7.38e^5 \pm 6.96e^4$, and mean post-test = $5.91e^5 \pm 1.07e^5$. The increase in the breath signal during the test phase was significant when compared with both the pre- and post-test levels ($p < 0.02$), whereas the pre- and post-test signal levels did not differ appreciably ($p < 0.30$) (Dunnett's multiple comparisons test and also paired *t*-test analysis).

The breath signal was lower across all three experimental phases in the T2DM subjects, but it retained the same profile as that present in the healthy subjects, and the results of statistical elaboration were alike. There were significant differences among the three experimental phases ($F(2,4) = 18.02$, $p < 0.01$): mean pre-test = $4.32e^4 \pm 8.93e^3$, mean test = $5.84e^4 \pm 1.22e^4$, and mean post-test = $3.93e^4 \pm 1.11e^4$. The

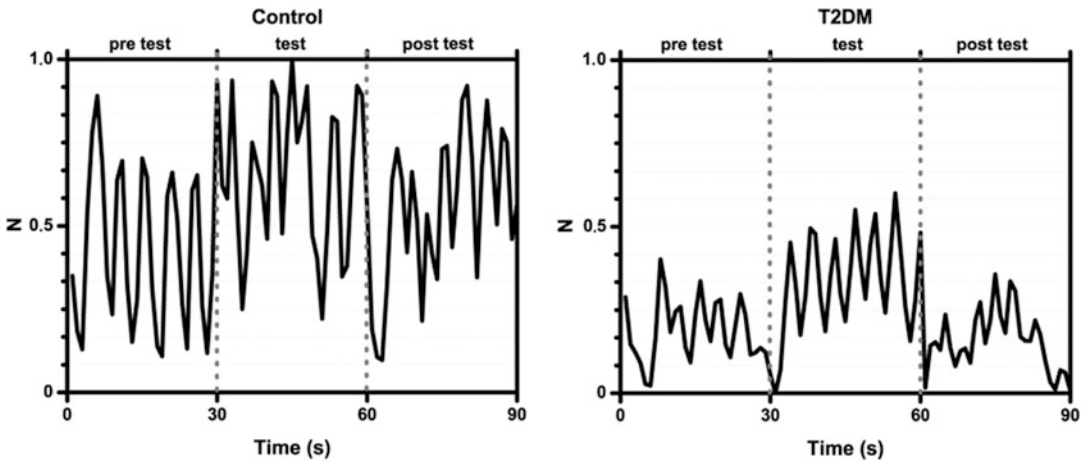

Fig. 35.2 Normalized breath signal in a representative control subject compared with that in a diabetic (T2DM) subject, recorded through the consecutive phases of the experiment; pre-test: signal of normal free breathing, test: breathing signal during cognitive test; post-test: recovery breathing signal after the cognitive test. Normalization formula is X = (xi − xi,j min)/(xi,j max − xi, j min)

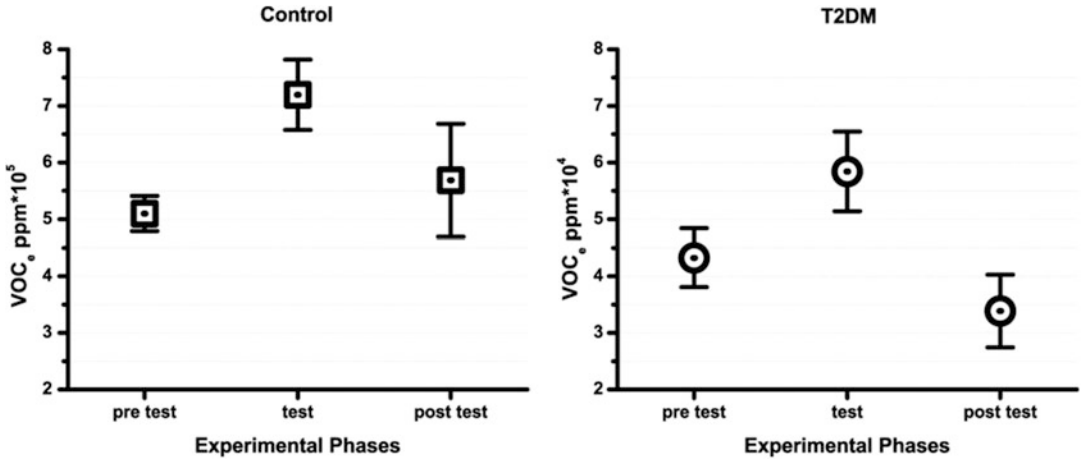

Fig. 35.3 Comparison of the mean exhaled VOC in the control versus diabetic (T2DM)) subjects in the three experimental phases

increase in the breath signal during the test phase was appreciably different from both pre-test and post-test phases ($p < 0.03$), whereas the latter two phases did not differ from each other ($0.05 < p < 0.06$) (Dunnett's multiple comparisons test and *t*-test).

Comparison of the average levels of exhaled VOCe of the tree experimental phases between the control and T2DM subjects demonstrates that the rebound in the recovery phase after the cognitive task in the T2DM patients was faster and reached lower values (Fig. 35.3). The percentage variation difference between the test and post-test values between the control and T2DM subjects was significant; means: 9.27 ± 11.69 vs. 23.00 ± 10.32, respectively (ANOVA; $F(2,4) = 12.85$; $p < 0.02$). This difference was calculated for the test value as: [(test value minus pre-test value)/(pre-test value)]*100 and for the post-test value as: [(post-test value minus pre-test value)/(pre-test value)]*100.

4 Discussion

Cognitive deficits and hippocampal atrophy, features that are shared with aging and dementia, have been described in T2DM. Moreover, these features are associated with obesity, hypertension, dyslipidemia, hypothalamic pituitary adrenocortical axis abnormalities, microvascular alteration, neural slowing, increased cortical atrophy, microstructural abnormalities in white matter tracts, changes in brain neurometabolites which have to do with in mental, learning, and memory deficits, motor slowing, decrements of attention and executive functioning (McCrimmon et al. 2012; Williamson et al. 2012; Bruehl et al. 2009; Gold et al. 2007). Additionally, in T2DM oxidative stress plays a crucial role associated with chronic hyperglycemia and inflammation (West 2000). Breath analysis enables to define the level of oxidative stress being reflected in the concentration of volatile organic compounds (VOCs) excreted in the breath (Phillips et al. 2004). Intriguingly, we found in the present study that total concentration of exhaled VOCe in the breath of T2DM patients was ten-fold lower than that in control subjects. This result is interesting since, although numerous studies have been devoted to identify biomarkers characteristic of T2DM patients (e.g., Phillips et al. 2004), a quantitative measurement of VOCe in real-time monitoring has not yet been thoroughly investigated. However, this result is in line with the qualitative breath methylated alkane contour (BMAC) profile obtained in T1DM, T2DM, and in normal subjects in a previous analytical study (Phillips et al. 2004). The quantitative reduction of total VOCe measured in the breath of T2DM patients is due likely to metabolic alterations stemming from insulin resistance or, conversely, from pharmacological treatment of these patients. We speculate that reduction in VOCe is connected with reduced metabolic function in the light of insulin resistance that decreases the quantity of glucose being utilized. The resolution of this issue would require the employment of a qualitative analytical assay of breath content in T2DM during physical or cognitive performance.

In the present study we also analyzed the profile of the breath signal while performing a cognitive task. Interestingly, the breath signal in both control and T2DM subjects showed the same pattern. The finding confirms our hypothesis that cognitive taxing of the brain, by increasing metabolism, would be reflected in increased concentration of exhaled VOCe. An increase in exhaled VOCs, e.g., isoprene, has been previously observed in psychological stress (Conkle et al. 1975). Increases in VOCe could be related to increased insulin receptor expression in the hippocampus which is heavily involved with the cognitive processing. This presumption also provides an explanation for the effects on hippocampal-dependent tasks of insulin resistance seen in previous studies (Gold et al. 2007; Bruehl et al. 2009; McCrimmon et al. 2012). Our reasoning and explanation above outlined seem supported by the alterations in VOCe in the post-test phase observed in the present study. A greater decrease below the baseline level of normal breathing of VOCe in the recovery phase from the cognitive task in T2DM patients, compared with healthy subjects, suggests a greater neural fatigue in the patients caused by the task.

In summary, the major findings of this preliminary study are as follows: total concentration of VOCe is reduced in diabetic patients, the profile of VOCe changes in response to a cognitive task is similar in both healthy and diabetic subjects, but diabetic patients show a greater decrease below the baseline pre-task level in the post-task VOCe. The study shows that the recording system employed is suitable for the continuous real-time non-invasive monitoring of VOCs in exhaled breath in humans. Interestingly, VOCe measurement enables the detection of a level of cognitive effort in both healthy and sick subjects. Thus, analysis of exhaled breath content might offer a novel diagnostic and therapeutic non-invasive approach in metabolic and neurodegenerative derangements.

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

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Exhaled Nitric Oxide in Specific Inhalation Challenge

36

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Abstract

Exhaled nitric oxide (eNO) is a biological mediator in human lungs and can be measured easily in exhaled air. Increasing eNO concentrations after specific inhalation testing (SIT) have been described for subjects with occupational asthma. Nevertheless, interpreting eNO concentrations after SIT is still a challenge because eNO concentrations depend on various confounding factors. In this study, 24 women and 43 men with suspected occupational asthma were examined by a questionnaire, physical examination, routine laboratory testing, skin prick testing (atopy: at least one wheal reaction >3 mm), lung function including methacholine testing, and SIT with various occupational allergens. Exhaled NO was measured before SIT (t0), 2 h (t1) and 20–22 h (t2) afterwards (NIOX Flex, Aerocrine, Sweden). At baseline we observed significantly lower eNO concentrations in smokers than in non-smokers and in non-atopics than in atopics (significant only in SIT non-responders). In the SIT non-responders (n = 45), eNO concentrations showed no change after SIT (t0: 16.0, t1: 12.3, t2: 16.0 ppb). In the SIT responders (n = 22), eNO was elevated significantly at t2 (t0: 22.9, t1: 19.9, t2: 42.0 ppb). In addition to positive responder status and measuring time, missing atopy and exposure to isocyanates were the essential factors leading to increased eNO concentrations. We conclude that the measurements of eNO after SIT may provide valuable information concerning the allergenic status of a patient.

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_36,

Keywords
Exhaled air • Exhaled nitric oxide • Occupational asthma • Inhalation testing • Methacholine testing • Isocyanates

1 Introduction

Exhaled nitric oxide (eNO) is increased in eosinophilic inflammation in human lungs. Exhaled NO can be measured easily in exhaled air (Dweik et al. 2011; Szefler et al. 2012). Increased concentrations of eNO are described, e.g., in asthma and therefore eNO may be used in the diagnosis of this disease if objective evidence is needed (Dweik et al. 2011; Quirce et al. 2010). Exhaled NO is modified by several factors, e.g., atopy, which increases, and smoking, which decreases eNO concentrations (Dweik et al. 2011).

Occupational asthma (OA) is often diagnosed by specific inhalation testing (SIT), but the studies on the influencing factors of eNO in SIT are rare. Bronchial hyperresponsiveness (Cruz and Munoz 2012; Lemiere et al. 2012; Allmers et al. 2000; Barbinova and Baur 2006), atopy and specific IgE (Allmers et al. 2000), smoking (Allmers et al. 2000; Baur and Barbinova 2005), and steroid therapy (Allmers at al. 2000; Baur and Barbinova 2005) were described as modifying factors. It was the aim of this study to analyze confounding factors for eNO in SIT with different occupational allergens.

2 Methods

The study was performed in conformity with the Declaration of Helsinki of the World Medical Association and the protocol was approved by a local Ethics Committee.

Subjects were examined in our outpatient clinic for occupational asthma by a questionnaire, physical examination, routine laboratory testing, skin prick testing (SPT, occupational and common environmental allergens; atopy: at least one wheal reaction >3 mm), and lung function testing. Smoking status was classified in non-smokers (never-smokers or ex-smokers) and current smokers. Latency was defined as the time between the beginning of exposure to the onset of symptoms suggestive of OA. Asthma medication was withheld for at least 12 h before methacholine testing and 24 h before SIT. Bronchial hyperresponsiveness was measured with methacholine (Baur et al. 1998). SIT was conducted according to the German guidelines and evaluated by spirometry and body plethysmography (decrease of $FEV_1 \geq 20$ % or increase of sRt of $\geq$100 % to $\geq$2 kPa*s) (Gonsior et al. 2002). Exhaled NO was measured according to the ATS-recommendations at 50 mL/s before SIT (t0), 2 (t1) and 20–22 h (t2) after SIT (NIOX Flex, Aerocrine, Sweden).

Allergens were categorized in three groups according to the size of the allergens (high-molecular weight allergens = HMA-group, low-molecular weight allergens = LMA-group, isocyanates = I-group). We analyzed the absolute concentrations of eNO at different time points (before SIT (baseline) = t0, 2 h after SIT = t1 and 22–24 h after SIT = t2), and the difference of eNO concentrations between t0 and t2 using descriptive statistic measures (median and interquartile range). Group differences were tested with Wilcoxon rank-sum tests and Kruskal-Wallis tests. P-values below 0.05 were declared as significant.

3 Results

3.1 Subjects

We included 67 patients, 24 women and 43 men (HMA-group: n = 16, LMA-group: n = 21, I-group: n = 28, others: n = 2) (Table 36.1). Exposure resulted from work in the chemical industry, as painters or hairdressers, and in a few cases from other occupations (e.g., farmer, miner, or technician). Median time since

Table 36.1 Description of the study population

	Total		HMA-group: high molecular (latex, flour, animal hair, moulds)		LMA-group: low molecular (hairdressers' substances, platinum, rhodium)		I-group: isocyanates	
	Women	Men	Women	Men	Women	Men	Women	Men
	N = 24	N = 43	N = 4	N = 12	N = 11	N = 10	N = 8	N = 20
Median (interquartile range)								
Age (year)	43 (32–50)	42 (31–52)	40 (33–48)	46 (32–57)	40 (25–47)	32 (27–43)	46 (40–50)	44 (35–53)
IgE (kU/L)	60 (17–143)	60 (17–206)	61 (58–359)	113 (38–324)	79 (21–137)	105 (17–702)	31 (10–152)	42 (14–83)
Latency (years)	6.0 (1.0–9.5)	5.0 (2.0–13.0)	8.0 (4.0–13.5)	9.5 (5.0–20.5)	6.0 (3.0–14.0)	1.0 (1.0–3.0)	1.0 (1.0–7.0)	4.5 (2.5–14.5)
Cessation of exposure (years)	1.0 (0.0–2.0)	0.5 (0.0–2.0)	1.5 (0.5–2.5)	0.0 (0.0–1.5)	1.0 (0.0–1.0)	0.0 (0.0–2.0)	1.0 (0.0–3.0)	1.0 (0.0–1.5)
FEV_1% pred	96 (88–106)	92 (85–106)	107 (100–111)	86 (81–97)	90 (86–105)	95 (85–105)	92 (90–106)	98 (91–108)
N (%)								
Non-smokers	14 (58 %)	29 (67 %)	4 (100 %)	9 (75 %)	3 (27 %)	5 (50 %)	6 (75 %)	15 (75 %)
Current smokers	10 (42 %)	14 (33 %)	0 (0 %)	3 (25 %)	8 (73 %)	5 (50 %)	2 (25 %)	5 (25 %)
Atopic persons	10 (42 %)	20 (47 %)	4 (100 %)	8 (67 %)	4 (36 %)	4 (40 %)	2 (25 %)	8 (40 %)
Current therapy								
With steroids	6 (25 %)	16 (37 %)	1 (25 %)	6 (50 %)	3 (27 %)	2 (20 %)	2 (25 %)	7 (35 %)
With bronchodilators	13 (54 %)	18 (42 %)	3 (75 %)	9 (75 %)	6 (55 %)	4 (40 %)	4 (50 %)	4 (20 %)

cessation of exposure was nearly identical in all exposure groups (about 1–2 years).

Although there was a large range, median age was comparable between groups. The highest proportion of atopics was diagnosed in the HMA-group, whereas in both other groups there were similar proportions. In both genders, the amount of atopics was similar. Non-smokers were predominantly seen in the HMA- and I-groups, whereas smokers dominated in the LMA-group. The HMA group showed the highest proportion of subjects on current therapy with bronchodilators, whereas steroids were used in all groups in nearly identical proportions.

3.2 Baseline eNO Concentrations

Baseline median eNO concentrations in the subjects with negative SIT (non-responders) were lower (13.0 ppb) than in persons with positive SIT (responders; 22.9 ppb), but the difference was not statistically significant (Table 36.2). While in the non-responders, eNO baseline concentrations >25 ppb were not seen, median eNO concentrations >25 ppb were found in several subgroups of responders: non-smokers, HMA-group, bronchial hyperresponsiveness, cessation of exposure <1 year and latency period >3 years (Table 36.2).

Baseline eNO was lower in current smokers than in non-smokers ($p < 0.05$), and lower in non-atopics than in atopics. In the non-responders, this difference was significant ($p < 0.05$). In the responders, latency was associated positively and period of exposure cessation was associated negatively with the eNO baseline concentrations ($p < 0.05$). Gender, classification of allergens, bronchial hyperresponsiveness, and current therapy did not modify eNO concentrations.

3.3 eNO Concentrations After SIT

In the non-responders ($n = 45$), eNO showed a tendency to lower concentrations at t1 and a small increase at t2 (median eNO: 13.0, 12.3, 16.0 ppb), only had the non-smokers slightly higher values at t2 (Table 36.2). The variation of the median eNO concentrations between t0, t1 and t2 was mostly <5 ppb. In general, median eNO concentrations in the non-responders showed no change after SIT and remained below 25 ppb, with the exception of atopics (t2: 25 ppb) and the HMA-group (t2: 27 ppb).

Median eNO concentrations were elevated at t2 in the responders (16.0 vs. 41.0 ppb, $p = 0.0012$). Like that in the non-responders, eNO at t1 showed a tendency to lower concentrations, although the non-atopics, subjects after isocyanate challenge, or on current therapy with steroids showed a small increase of eNO from baseline at t1. None of these increases were significant.

The subjects from I-group showed higher eNO concentrations at t2 than those of the other exposure groups (81.5 vs. 27.2 and 33.0 ppb, respectively). Due to intersubject variability, this difference was insignificant. Similarly, there was a trend for higher eNO concentrations at t2 in the non-smokers, non-atopic subjects without bronchial hyperresponsiveness, and those with longer latency periods and shorter exposure cessation. None of these differences were significant. In the responders, a median increase of eNO was about 83% between t0 and t2 (22.9–42.0 ppb). In addition to positive responder status and measuring time, missing atopy ($p = 0.0091$), shorter latency period ($p = 0.037$), and exposure to isocyanates ($p = 0.0079$) were the essential factors leading to increased eNO concentrations. Both maximal decrease of FEV_1 (i.e., severity of the obstructive reaction in SIT) and baseline eNO-concentrations were not associated with an increase of eNO (Tables 36.2 and 36.3).

4 Discussion

Our results show that the absolute median eNO concentrations after SIT are modified mainly by the result of SIT and a measuring time of 22–24 h after SIT. Furthermore, an increase of eNO concentrations between baseline and t2 in responders seems to be modified by further factors. Higher baseline eNO concentrations

Table 36.2 eNO concentrations before specific inhalation challenges (SIT)(baseline), 2–4 h and 22–24 h after SIT (n = 67)

		eNO (ppb) Baseline			eNO (ppb) 2–4 h after SIT			eNO (ppb) 22–24 h after SIT		
SIT		n	Median	Interquartile range	n	Median	Interquartile range	n	Median	Interquartile range
Negative		45	13.0	(9.0; 27.9)	45	12.3	(8.0; 22.7)	40	16.0	(9.6; 31.2)
Positive		22	22.9	(11.5; 36.1)	22	19.9	(10.2; 32.1)	20	42.0	(23.2; 82.5)
P value				0.0987			0.1043			0.0012
Negative	Women	20	16.0	(10.2; 29.8)	20	14.9	(7.6; 25.1)	19	13.5	(9.0; 40.1)
	Men	25	11.9	(8.6; 27.5)	25	10.0	(8.0; 21.4)	21	18.0	(10.4; 25.0)
	P value			0.4245			0.5826			0.6634
Positive	Women	4	12.3	(8.8; 15.9)	4	8.5	(5.9; 17.6)	4	53.2	(22.5; 88.2)
	Men	18	26.3	(14.0; 40.8)	18	24.4	(14.7; 34.3)	16	42.0	(23.2; 82.5)
	P value			0.0657			0.0979			0.9635
Negative	Non-smokers	29	16.0	(9.4; 29.8)	29	18.0	(8.0; 24.3)	28	19.9	(10.3; 42.1)
	Current smokers	16	10.7	(7.2; 14.5)	16	9.4	(7.6; 14.1)	12	11.1	(9.3; 19.7)
	P value			0.0422			0.1286			0.1518
Positive	Non-smokers	14	29.6	(19.7; 44.0)	14	24.4	(15.0; 44.0)	12	62.2	(26.8; 86.0)
	Current smokers	8	10.8	(7.8; 17.2)	8	8.4	(4.2; 27.8)	8	27.0	(19.9; 55.8)
	P value			0.0029			0.0420			0.2383
Negative	Non-atopic	27	11.0	(8.0; 16.3)	27	9.0	(7.5; 18.0)	23	12.1	(9.0; 21.7)
	Atopic	18	23.7	(11.3; 29.0)	18	22.1	(10.0; 32.0)	17	25.0	(14.3; 40.1)
	P value			0.0254			0.0067			0.0718
Positive	Non-atopic	10	19.2	(11.5; 32.0)	10	19.9	(6.7; 32.1)	8	67.4	(33.0; 88.9)
	Atopic	12	24.9	(12.1; 44.1)	12	22.9	(12.7; 32.5)	12	27.9	(17.0; 70.2)
	P value			0.3812			0.6744			0.2379
Negative	HMA-group[a]	9	23.0	(8.0; 31.5)	9	22.0	(8.9; 25.6)	6	27.7	(18.0; 63.4)
	LMA-group[b]	13	11.0	(8.6; 16.0)	13	9.7	(8.0; 15.8)	13	12.1	(9.7; 14.3)
	I-group[c]	21	16.0	(9.4; 27.9)	21	13.9	(8.0; 21.4)	20	19.4	(9.7; 36.1)
	Others	2	7.3	(3.1; 11.5)	2	5.5	(3.0; 8.0)	1	2.0	(2.0; 2.0)
	P value			0.3745			0.2374			0.1315
Positive	HMA-group[a]	7	25.4	(14.0; 36.1)	7	17.5	(14.7; 30.6)	7	27.6	(13.0; 44.2)
	LMA-group[b]	8	20.6	(11.5; 29.6)	8	19.9	(8.4; 33.2)	6	33.0	(21.0; 56.9)
	I-group[c]	7	18.7	(6.1; 52.1)	7	25.0	(5.0; 44.0)	7	81.5	(54.6; 96.3)
	P value			0.6701			0.9224			0.1410

(continued)

Table 36.2 (continued)

SIT		eNO (ppb) Baseline			eNO (ppb) 2–4 h after SIT			eNO (ppb) 22–24 h after SIT		
		n	Median	Interquartile range	n	Median	Interquartile range	n	Median	Interquartile range
Negative	No	26	14.3	(9.0; 27.9)	26	11.5	(8.0; 21.4)	23	16.9	(9.0; 40.1)
	Yes	18	13.5	(9.1; 28.0)	18	13.1	(8.0; 25.6)	16	16.5	(9.6; 30.0)
	P value			0.9577			0.6494			0.9606
Positive	No	4	22.1	(15.1; 52.0)	4	19.9	(12.5; 44.4)	4	81.8	(52.8; 89.9)
	Yes	16	25.8	(11.6; 38.5)	16	24.4	(8.7; 33.2)	14	42.0	(26.3; 81.5)
	P value			1.0000			0.9635			0.4420
Negative latency	≤3 years	15	11.9	(8.6; 31.5)	15	9.7	(6.1; 24.3)	14	13.6	(9.0; 30.3)
	>3 years	30	13.5	(9.4; 27.9)	30	13.5	(8.0; 22.7)	26	17.5	(10.2; 32.0)
	P value			0.7612			0.3129			0.3429
Positive	≤3 years	12	15.9	(9.7; 24.3)	12	14.8	(5.9; 26.7)	10	55.8	(21.0; 96.3)
	>3 years	10	35.3	(24.4; 44.0)	10	30.2	(15.0; 44.0)	10	33.9	(25.4; 81.5)
	P value			0.0169			0.0804			0.5789
Exposure cessation Negative	≤1 year	30	14.8	(8.6; 27.9)	30	12.6	(8.0; 23.3)	26	16.0	(10.2; 30.3)
	>1 year	15	12.9	(9.0; 29.0)	15	10.0	(7.2; 22.7)	14	15.5	(9.0; 44.0)
	P value			0.7886			0.9668			0.8064
Positive	≤1 year	19	25.4	(14.0; 40.8)	19	20.5	(10.7; 34.3)	17	44.2	(26.3; 81.5)
	>1 year	3	10.0	(9.4; 11.5)	3	10.0	(3.4; 25.0)	3	21.0	(6.2; 96.3)
	P value			0.0404			0.1909			0.4789
Current therapy with steroids Negative	No	33	11.9	(8.6; 27.9)	33	10.0	(8.0; 21.4)	30	13.9	(9.7; 40.1)
	Yes	12	16.0	(10.3; 25.5)	12	16.0	(8.0; 24.2)	10	19.9	(9.1; 25.0)
	P value			0.5134			0.4423			0.8356
Positive	No	12	22.6	(10.1; 38.5)	12	17.0	(10.1; 29.0)	10	32.6	(18.7; 80.1)
	Yes	10	22.9	(13.0; 34.5)	10	27.8	(15.0; 44.0)	10	55.8	(27.6; 83.5)
	P value			0.9743			0.2829			0.2474
Current therapy with bronchodilators Negative	No	28	10.8	(8.3; 27.7)	28	9.7	(7.4; 20.6)	25	12.9	(9.0; 32.0)
	Yes	17	16.0	(11.5; 28.0)	17	15.8	(9.9; 25.6)	15	18.0	(12.1; 30.3)
	P value			0.0683			0.0377			0.1420

Positive	No	8	22.6	(14.4; 46.5)	8	17.6	(10.1; 30.2)	7	39.7	(21.0; 83.5)
	Yes	14	22.9	(11.5; 34.5)	14	22.2	(10.7; 34.3)	13	44.2	(26.3; 81.5)
	P value			0.7140			0.6167			0.8775
Max. FEV_1-decrease after SIT										
Negative	≤30 %	45	13.0	(9.0; 27.9)	45	12.3	(8.0; 22.7)	40	16.0	(9.6; 31.2)
Positive	≤30 %	13	24.4	(11.5; 36.1)	13	29.7	(10.7; 34.3)	13	44.2	(27.6; 83.5)
	>30 %	9	19.7	(13.0; 27.2)	9	15.0	(6.7; 20.5)	7	26.3	(18.7; 81.5)
	P value			0.5557			0.1443			0.5884
Exhaled NO before SIT										
Negative	≤25 ppb	31	10.2	(8.0; 14.0)	31	9.0	(7.2; 12.9)	27	10.4	(9.0; 18.0)
	>25 ppb	14	34.1	(28.0; 49.2)	14	25.0	(21.4; 48.7)	13	44.0	(25.0; 54.8)
	P value			–			<0.0001			<0.0001
Positive	≤25 ppb	12	12.3	(9.7; 19.2)	12	10.5	(5.9; 19.0)	11	26.3	(13.0; 56.9)
	>25 ppb	10	38.5	(32.0; 52.1)	10	31.4	(28.3; 63.8)	9	81.5	(39.7; 88.4)
	P value			–			0.0008			0.0381

[a]HMA-group: high molecular weight allergens (latex, flour, animal dander, moulds);
[b]LMA-group: low molecular weight allergens (hairdressers' substances, platinum, rhodium)
[c]I-group: isocyanates

Table 36.3 Increase of eNO concentrations between baseline (t0) and 22–24 h after SIT (t2) in responders (n = 20)

Δ eNO [ppb]	n	Median	Interquartile range	P value
Gender				
Women	4	37.4	(13.0; 73.1)	0.062[d]
Men	16	4.7	(−0.5; 36.5)	
Smoking status				
Non-smoker	12	4.7	(0.0; 41.0)	0.720[d]
Current smoker	8	13.0	(3.9; 43.7)	
Skin prick testing				
Non-atopic	8	44.7	(13.0; 73.1)	0.0091[d]
Atopic	12	4.1	(−0.5; 9.6)	
Classification of allergens				
HMA-group[a]	7	0.0	(−1.0; 4.4)	0.0079[e]
LMA-group[b]	6	12.2	(−1.1; 35.5)	
I-group[c]	7	44.4	(12.6; 61.4)	
Bronchial hyperresponsiveness				
No	4	33.2	(2.5; 73.1)	0.454[d]
Yes	14	10.4	(0.0; 37.5)	
Latency				
≤3 years	10	40.0	(12.6; 61.4)	0.0037[d]
>3 years	10	1.9	(−1.0; 4.9)	
Exposure cessation				
≤1 year	17	8.1	(0.0; 37.5)	0.990[d]
>1 year	3	11.0	(−3.2; 84.8)	
Current therapy with steroids				
No	10	6.3	(0.0; 12.6)	0.305[d]
Yes	10	24.4	(3.7; 51.8)	
Current therapy with bronchodilators				
No	7	4.9	(−1.1; 44.4)	0.497[d]
Yes	13	12.6	(3.7; 37.5)	
Max. FEV_1-decrease				
≤30 %	17	8.1	(0.0; 37.5)	0.990[d]
>30 %	3	11.0	(−3.2; 84.8)	
Exhaled NO before SIT				
≤25 ppb	11	12.60	(0.00; 51.80)	0.617[d]
>25 ppb	9	4.90	(0.00; 37.54)	

[a]HMA-group: high molecular weight allergens (latex, flour, animal dander, moulds)
[b]LMA-group: low molecular weight allergens (hairdressers' substances, platinum, rhodium)
[c]I-group: isocyanates
[d]Wilcoxon rank-sum test
[e]Kruskal-Wallis test

were repeatedly described in atopics in previous studies, a finding that was reproduced in the present study. Interestingly, increasing eNO concentrations after SIT were measured predominantly in non-atopic responders. This contrasts somewhat with previous findings (Allmers et al. 2000; Barbinova and Baur 2006), but the numbers of responders were much lower in those studies. The increase of eNO concentrations was important especially after isocyanate challenges. This is an unexpected finding which points to eosinophilic inflammation in isocyanate asthma, as reported earlier (Niimi et al. 1996). Whether there is a true

difference between high and low molecular weight substances has to be corroborated in higher numbers of study participants.

A negative association between latency period and eNO increase is difficult to explain. Among other hypotheses, one might assume that host factors are responsible for a quicker development of disease which is also reflected by higher eNO increases after challenges. Different quantitative and qualitative exposure indices represent further explanations.

Baseline eNO concentrations were modified negatively by smoking status, but smoking did not modify the increase of eNO concentrations after SIT. Similar results were reported by others (Allmers et al. 2000). This is important for clinical practice as challenges may be interpreted independent of the smoking status. Median increase of eNO in responders was about 19 ppb (82%) in the present study. Pedrosa et al. (2012) found 17 ppb (25%) and Barbinova and Baur (2006) found 16 ppb (106 %). This suggests that increases of eNO concentrations should be expressed in the absolute and not relative values.

No association were found between increases of eNO concentrations after SIT and gender, bronchial hyperresponsiveness, exposure cessation, and therapy. These observations have to be interpreted cautiously due to the low numbers of observations. This is especially true for subgroups, e.g., responders with and without hyperresponsiveness. Barbinova and Baur (2006) reported a higher increase of eNO after SIT in responders with hyperresponsiveness, but a detailed analysis of responders with ($n = 8$) and without ($n = 3$) hyperresponsiveness with respect to eNO increases was not provided.

There was a trend for a higher increase of eNO concentrations in subjects with asthma medication, which may be interpreted as higher disease severity. Similarly, Allmers at al. (2000) were not able to detect a relationship between medication and an increase of eNO concentrations, whereas Baur and Barbinova (2005) described only borderline changes in eNO concentrations after SIT in nine steroid treated latex sensitised subjects. According to our study, the effects of steroid medication should be limited. The interpretation of test results should be possible if medication is withheld for short periods.

It cannot be excluded that the lacking modifying effects on eNO concentrations after SIT of gender, duration of exposure cessation, baseline eNO concentrations, and maximal FEV_1 decrease after SIT may be due to the low numbers of subjects in the subgroups and a high intersubject variability. Our results were calculated with median eNO concentrations. Due to the large variability, one should be cautious to transfer the results to individual cases. Thus, we consider eNO measurements in SIT primarily as a confirmation test. It is one of the major results of this study that, in contrast to expectations, eNO concentrations appreciably increased in isocyanate asthma. It is not possible to separate quantitative from qualitative causative factors. If the largest fall of FEV_1 is taken as a surrogate for quantitative exposure, we did not find a relevant effect on eNO. Thus, one may hypothesize that LMA and especially isocyanates are inducing a higher degree of eosinophilic airway inflammation than HMA.

Whereas one of the strengths of our study was the inclusion of different occupational allergens, the main limitation was a small number of cases. Some confounding factors that were calculated to be non-significant could become significant in larger number of cases.

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

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Integration of Simulated Multipotential Signals: The Role of Integration Window Width and of the Number of Spikes

37

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Abstract

Electrical signals recorded from nerves/muscles represent the fundamentals for experimental data analysis including an assessment of respiratory motor output. The present work, based on theoretical model, is focused on the linearity and variability of rectified and integrated electroneurogram (ENG)/electromyogram (EMG) signals in relation to the frequency of spike incidence and moving average window width used for processing of signals. Our simulations of multipotential signals (multiunit action potentials) originating from an overlapping of four single units with phase shifts firing at two frequencies demonstrates that (1) integrated ENG/EMG signals are only approximately linearly proportional to the frequency of action potentials in the superposition – multipotential and (2) the width of the moving average window strongly influences the range (dispersion) of integrated values. Better quality of EMG recordings, a higher number of action potentials within the multipotential signals, and a wider width of the moving average window increase the accuracy of integrated ENG/EMG values during processing of motor output signals.

Keywords

Action potential • EMG • ENG • Moving average • Multipotential • Superposition

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_37,

1 Introduction

Nerves conduct signals in the form of action potentials. Action potentials represent an essential element of encoding and transmission of information in neural systems. They also represent the first stage in the cascade of triggering of muscle contraction. It is generally assumed (including the modeling of nerve signaling) that the compound nerve activity or multipotential is a linear superposition of single fiber action potentials (Baker and Lemon 1995).

The electromyogram (EMG) usually represents a complex electrical biosignal, a result of superposition of action potential trains recorded from muscle fibers located near the electrode and generated by active motor units (Barrett et al. 2012). The number of muscle fibers in the motor unit ranges widely across human and animal muscles (Merletti and Farina 2009). Muscle fibers of each motor unit are intermingled with fibers of other motor units, so fibers belonging to several different motor units are close to each other (Buchtal et al. 1957). The assumption of linearity of summation of their activation is crucial for evaluation of intensity of the entire muscle electrical activity, for decomposition of multiunit EMG, for computer simulations, and for mathematical models.

In this chapter, we studied the linearity of superpositions of action potentials from a few motor units putatively recorded at the electrode using a computer theoretical model. We hypothesized that moving average of rectified superposition of action potentials is not perfectly linear and that its variability and dispersion depend on the frequency of action potentials and on the width of integrative (moving average) windows.

2 Methods

All procedures were performed in accordance with the laws, rules, and regulations of Slovakia and the EU. The Ethics Committee of Jessenius Faculty of Medicine in Martin, Slovakia approved the protocols. The model consisted of five waveforms. Four of these waveforms simulated single unit EMG signals and the fifth waveform represented the algebraic summation of these four single units (Fig. 37.1). A three phase shapes of action potential (AP), i.e., similar to those of single units, was chosen. It corresponded to the majority of *in vivo* recordings and lasted 5 ms (the 1st and the 3rd waveform) and 7 ms (the 2nd and the 4th waveform) (Day and Hulliger 2001; Dumitru et al. 1998). All waveforms were shifted in time from the first one. The second waveform started 4 ms, the third 1 ms, and the fourth 5 ms after the first one. Their frequency was 5–75 Hz (the 1st and 3rd waveforms) and 9–135 Hz (the 2nd and 4th waveforms) and corresponded to the occurrence of action potentials within weakly to extremely intensively stimulated muscles (McNulty et al. 2000; Burke 1981; Farmer et al. 1993) (Fig. 37.1). These frequencies stretch over the intervals of action potential firing rates for both, EMG and ENG of afferent and motor fibers. Adam and De Luca (2005) reported the mean action potential frequency of human vastus lateralis muscle during fatiguing isometric contractions at about 15 Hz. Nail et al. (1969) recorded discharges at peak frequencies of 197 impulses/s from epipharyngeal afferent fibers in anaesthetized, paralyzed cats.

The integrated waveform represented the rectified (absolute values) and averaged signal. Rectification was performed according to formula $f(x) = abs(x)$ and averaging to $f(x) = \Sigma x_i/i$, where x represented the particular samples of waveform, i represented the number of samples, and i was changing with window width. The moving average window was shifted in 0.01 ms steps during calculation of moving average waveform (integration). We employed three window widths – 1 s, 200 and 40 ms. The width of 1 s was taken as a standard reference value, the two other widths that comply with the time constants employed in real EMG/ENG signal processing were compared with it. The theoretical model was built and simulations were performed in a PC environment using MATLAB v. 7.5.

We tested the linearity of action potential integration for each of four single unit waveforms as

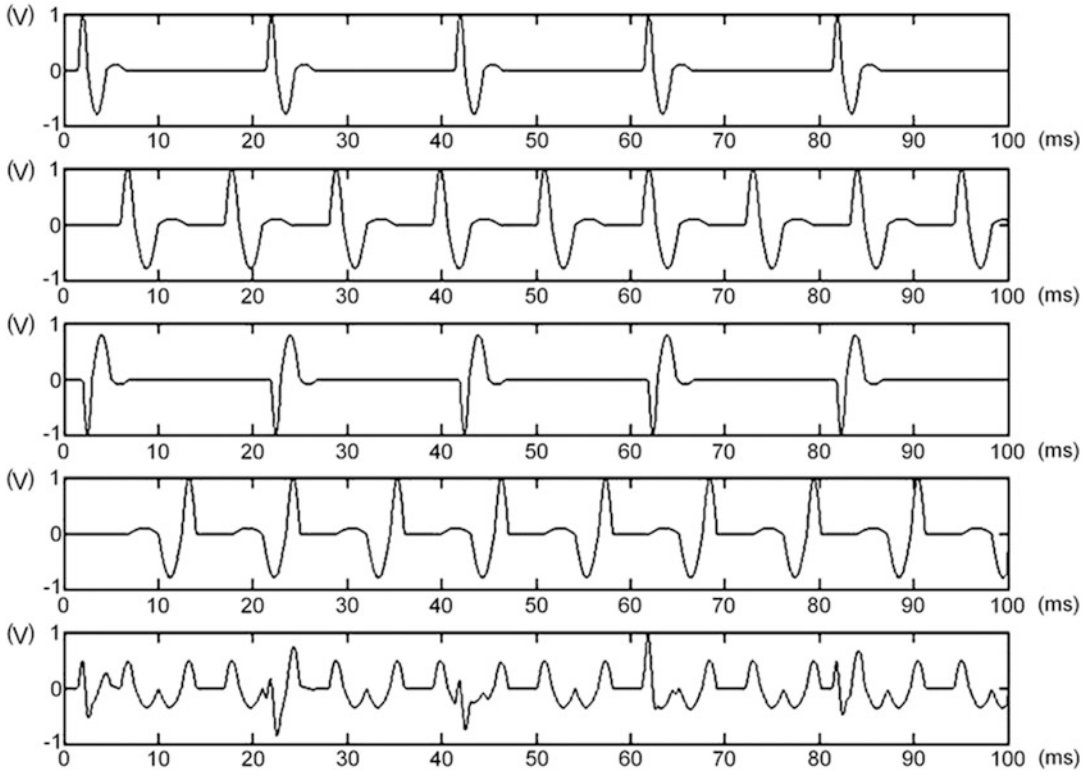

Fig. 37.1 Four waveforms of simulated single unit EMG signals with three phase shapes of action potentials (*top traces*). The *bottom waveform* represents the algebraic summation of the four single unit waveforms

well as for their superposition. The difference from exactly linear course of the moving average value with increasing frequencies of action potentials was determined as the maximum difference of actual modeled value from the course of linear regression. The frequency of action potentials was increased successively (the steps were 5 Hz for the 1st and 3rd and 9 Hz for the 2nd and 4th single unit waveforms) and the rectified and moving average values of all five waveforms were acquired and analyzed. Then, the superposed waveform was rectified and integrated using moving average window widths of 200 and 40 ms. Minimal and maximal values in the entire range of integration were found. The differences of maximum and minimum value representing the variability of signal were determined for both moving average window widths. Subsequently, the dispersion was calculated as values that differ most from the standard (integration of composed signal with the window width of 1 s – all action potentials were taken). This dispersion was expressed as a percentage of actual integrated values of superposed waveforms for both 200 and 40 ms windows.

In order to compare our modeled data with real EMG signals we recorded EMGs in experiments on cats using bipolar insulated fine-wire electrodes (see e.g., Poliacek et al. 2007, 2009b). Diaphragm, external oblique and/or transversal abdominal muscles, and upper esophageal sphincter were recorded and analyzed for this study. All EMGs were amplified, filtered (300–3,000 Hz; Grass Amplifiers), digitalized (12-bit multi-function plug-in ISA card, Dataq Instruments; sampling frequency of 10,000 Hz) and recorded by WinDaq v. 2.52 software (Dataq Instruments, Akron, OH). Experimental data were processed analogously to simulated ones. They were full wave rectified and moving averages with 200 and 40 ms window widths were calculated.

3 Results

At our 'standard' moving average window width of 1 s, the integrated values of four single unit waveforms increased linearly with the frequency of action potentials. However, moving average values (integrated signal) of the superposed waveform increased only approximately linearly with the frequency of the occurrence of action potentials even at this very long integration window (Fig. 37.2). The maximum difference from the optimum linear course of moving average values (with increasing frequencies of action potentials) was 0.011562 at the moving average value equaled to 0.537857 (the regression value was 0.549419). It occurred at integration of superposed signal of 65 spikes for the 1st and 3rd single unit spike trains and 117 spikes for the 2nd and the 4th ones. The linear regression dependence was described as 0.0014665 × number of spikes + 0.015613. The coefficient of linear regression r was 0.9994.

The average variability of moving average signals (the differences maximum minus minimum) found in waveforms of integrated signal was 3.2-fold lower with 200 ms moving average window than that with 40 ms moving average window (Fig. 37.3). The highest variability of moving average values of 0.172548 was found using 40 ms wide moving average window at a low interval of frequencies (5–9 Hz for the 1st and 3rd single unit spike trains and 10–18 Hz for the 2nd and 4th ones within the multipotential). The minimum variability at 40 ms window width was 0.051456 (at 65 spikes/s for the 1st and 3rd single unit waveforms and 117 Hz for the 2nd and 4th ones). Using 200 ms wide moving average window, the highest variability of the multipotential signal – 0.059115 was found at frequencies of 40 Hz (for the 1st and 3rd single unit spike trains) and 72 Hz (for the 2nd and 4th ones) and the lowest value – 0.013837 at 70 Hz (for the 1st and 3rd single unit spike trains) and 126 Hz (for the 2nd and 4th ones).

Dispersions were determined for 15 pairs of frequencies in the superposed signal (see methods). The maximum dispersion in moving averages at the lowest frequency of incidence of action potentials (5 and 9 Hz) was 259 % for

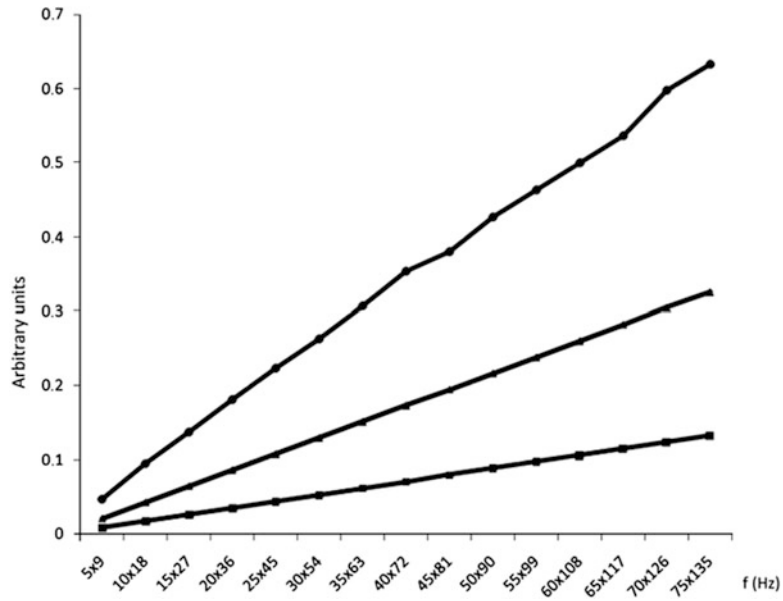

Fig. 37.2 Relation of integrated EMG signal values and the frequency of action potentials. '*Square*' *line* (*bottom*) – values of waveforms (1st and 3rd single unit trains) with 5 ms duration of action potentials, '*triangle*' *line* (*middle*) – values of waveforms (2nd and 4th single unit trains) with 7 ms duration of action potentials, '*circle*' *line* (*top*) – superposed waveform

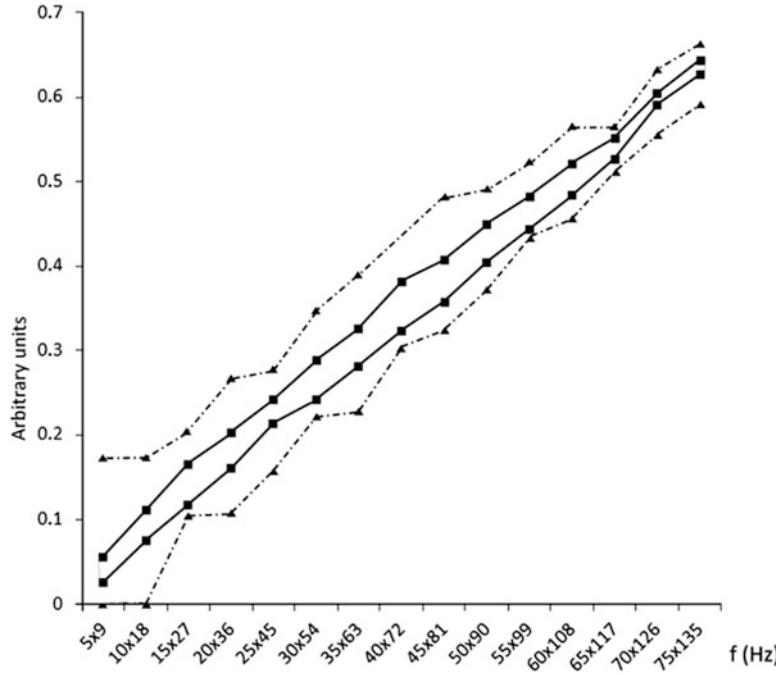

Fig. 37.3 Variability of moving average values related to different moving average window width (200 ms – *solid* (*squares*) *lines* interval, 40 ms – *broken* (*triangles*) *lines* interval) and frequency of action potentials

40 ms integration window width, while it reached only 17 % using 200 ms window. The dispersions decreased with increasing frequency of action potentials in the waveforms. At the highest simulated frequencies of action potentials (75 and 135 Hz) it stretched to 10.2 % for 40 ms window and 1.6 % for 200 ms window (Fig. 37.4).

Analysis of 11 sequences of real EMG recordings with putatively stable signal lasting 600–1,200 ms showed the dispersions of moving averages 12 ± 1 and 66 ± 4 % when signals were processed using 200 and 40 ms integration window width, respectively. The mean variability of moving averages of real EMGs was 6.0 ± 0.4 times higher when shorter (40 ms) integration windows were employed.

More than a 100 bursts of EMG activity of diaphragm, abdominal muscles, and upper esophageal sphincter during quiet breathing and cough were explored to determine the duration of EMG sequences with very high and near zero rectified signals. Such series, lasting around 5 ms, occurred in each of the examined burst of discharges. A few times, however, duration of these extreme segments lasted more than 8 ms and in few cases even more than 12 ms.

4 Discussion

Our analysis of superposition of action potentials within the multipotential of EMG/ENG processed by rectification and moving averaging demonstrated that (1) unlike the single unit waveforms the moving averages of multipotentials increased only approximately linearly with the frequency of action potentials and (2) the variability of moving average values and their relative differences from the optimum values (the dispersion of signal) markedly decreased with the number of spikes and the width of moving average window used.

Rectification and subsequent moving average processes accompanies the measurement of the level of EMG/ENG multiunit signals and motor

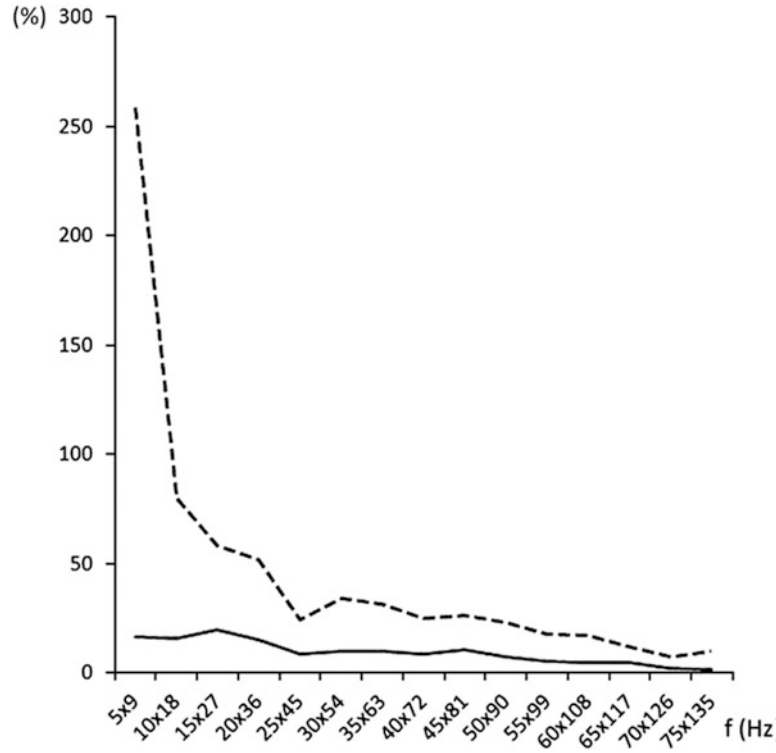

Fig. 37.4 Dispersion of moving average values decreases with increasing incidence of action potentials. 200 ms window width – *solid line*, 40 ms window width – *broken line*

and/or nerve drives (Merletti and Farina 2009; Day and Hulliger 2001; Poliacek et al. 2007, 2009b). Under optimum conditions, the values of moving average would increase linearly with the frequency of action potentials within the analyzed multipotential signal and their variability (maximum minus minimum detected under the same conditions) and dispersion (relative difference from the ideal value) would be very low. The compound nerve/muscle electrical activity is usually assumed to be a linear superposition of recorded action potentials (Baker and Lemon 1995); the activity being an algebraic summation of voltages from individual action potentials measured at the electrode (Day and Hulliger 2001). However, accidental variations occur in the integrated multipotential signal due to overlapping (and summation) of positive and negative components of individual action potentials. These overlapping can result in accidentally lower or higher level of moving average signal than the appropriate one (Baker and Lemon 1995; Day and Hulliger 2001). The regression analysis of four motor units multipotential simulated in this study confirmed the presence of a high linearity of the relationship of moving average values and of the number of spikes with the difference of optimum of simulated signals in a range of a few percentage points. The correlation coefficient r exceeded 0.99 suggesting a remarkably acceptable difference of simulated waveform progress from the real linearity. Similarly, EMG magnitudes determined from rectified signals were closely linearly related to motor unit activation in a well controlled experimental study of Day and Hulliger (2001).

Our simulations modeled constant motor drives lasting for 1 s that are optimally characterized by constant moving average value over all span of each individual activity. Employing 200 and 40 ms moving average window widths in the processing of multipotential we detected significant differences depending on actual positions of individual spikes (Figs. 37.3 and 37.4). The width of moving average window

significantly influences this variability of integrated values. An approximately three times higher variability in moving average values was found for the moving average window width of 40 ms compared to that for 200 ms window (see results and Fig. 37.3). Even a higher ratio (around 6) of moving average variability was found for 40 and 200 ms long integration windows during the analysis of real EMG recordings. Real EMGs within selected sequences lasting around 1 s cannot be assumed to be really constant. The motor drive may slightly vary (even when potentially constant activity segments were chosen) that would contribute to variability of moving average values and increased the variability caused by overlapping spikes. Our data, however, suggest that the moving averages obtained with longer integration constant are more accurate. The use of long time constants in EMG/ENG analysis will create problems concerning behaviors with short duration and the determining of temporal changes in the analyzed signals. While respiratory and cough related bursts of activities, e.g., in cats are long enough to employ time constants 100 and 200 ms in the processing of signal, other behaviors such as aspiration reflex last only several tens of ms (Poliacek et al. 2007, 2009a). To set the criteria for the optimum processing of short vs. long lasting signals goes beyond the goals of this study. However, a compromise between the accuracy of actual moving average value and the ability to detect time changes should be made. Our experimental data show that there are sequences of extreme values (very low or extremely high) lasting more than 12 ms in EMGs during vigorous motor drive of coughing (see results). Similar, but slightly shorter spans of very low or very high signal can be found even in our simulated multipotential (Fig. 37.1). The occurrence of these series significantly limits the choice of how short an integration window can be used. For example, using the 20 ms moving average window, the inaccuracy of processing of these series would be inacceptable; it would be much higher than the dispersion simulated in our study at low frequencies and 40 ms window width (Fig. 37.4).

The variability of moving average values and in particular their dispersion (relative difference from the optimum value) decreases apparently with the frequency of spikes (Figs. 37.2 and 37.3). The number of spikes within the multipotential depends on the number of active units recorded (the number of active nerve fibers or motor units and muscle fibers) and on the frequency of their discharges. Depending on the type of EMG electrode used and its location (the type and position in the muscle), it can record activity of a small (1–3), moderate (4–20), or large (more than 20) number of muscle fibers (Merletti and Farina 2009). Hypothetically, EMG recording of 4 muscle fibers with 15 action potentials/s each (the frequency that may induce tetanic contraction (Noto et al. 2011)) would correspond well to our simulation of 4 units at low firing frequencies (10 and 18 Hz) with the dispersion of moving average signal approximately 80 % at 40 ms window width (Fig. 37.4). Our simulations of a low number (4) of motor units proved that it is worth changing the position of the recording electrode if such situation is recognized during recording to have a higher number of spikes. A significant role of moving average window width is also demonstrated by the fact that under this condition, but when 200 ms window is used, the dispersion is only 15 %.

We conclude that a better quality of EMG/ENG recordings with a high number of active units, higher number of action potentials within the multipotential, and a wider width of the moving average window increase the accuracy and stability of ENG/EMG integration.

Acknowledgement Supported by the Slovak Grant Agency VEGA project No. 1/0038/09 and the Slovak Research and Development Agency under the contract No. APVV-0189-11.

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

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Functional and Topographic Concordance of Right Atrial Neural Structures Inducing Sinus Tachycardia

38

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Abstract

Cardiorespiratory autonomic control is in tight interaction with an intracardiac neural network modulating sinus node function. To gain novel mechanistical insights and to investigate possible novel targets concerning the treatment of inadequate sinus tachycardia, we aimed to characterize functionally and topographically the right atrial neural network modulating sinus node function. In 16 sheep 3-dimensional electroanatomical mapping of the right atrium was performed. In five animals additionally magnetically steered remote navigation was used. Selective stimulation of nerve fibers was conducted by applying high frequency (200 Hz) electrical impulses within the atrial refractory period. Histological analysis of whole heart preparations by acetylcholinesterase staining was performed and compared to the acquired neuroanatomical mapping.

We found that neural stimulation in the cranial part of the right atrium, within a perimeter around the sinus node area, elicited predominantly shortening of the sinus cycle length of -20.3 ± 10.1 % ($n = 80$, $P < 0.05$). Along the course of the crista terminalis atrial premature beats ($n = 117$) and atrial fibrillation ($n = 123$) could be induced. Catheter stability was excellent during remote catheter navigation. Histological

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_38,

work-up (n = 4) was in accord with the distribution of neurostimulation sites. Ganglions were mainly innervated by the dorsal right-atrial subplexus, with substantial additional input from the ventral right atrial subplexus. In conclusion, our findings suggest a functional and topographic concordance of right atrial neural structures inducing sinus tachycardia. This might open up new avenues in the treatment of heart rate related disorders.

Keywords
Autonomic nervous system • Cardiac electrophysiology • Cardiac innervation • Cardiorespiratory regulation • Electrophysiologic mapping • Remote magnetic navigation

1 Introduction

There is a tight interaction between cardiorespiratory autonomic control and the intrinsic cardiac autonomic nervous system (ICANS) modulating heart rate and rhythm (Tzeng et al. 2007; Drexel et al. 2013). It is now well established that the ICANS plays a critical role in inadequate sinus tachycardia (IST). Initially a surgical domain (Taketani et al. 2007), novel interventional strategies now rely on modulation of the ICANS by means of selective local ablation, in order to treat heart rate related disorders including IST (Femenía et al. 2012), neurally-mediated syncope (Pachon et al. 2005) and atrial fibrillation (Pokushalov et al. 2008). Anatomically and high-frequency-stimulation (HFS) guided ablation has been proposed, but identification of relevant intracardiac neural structures remains challenging (Pokushalov et al. 2009). In addition, catheter-based identification of intracardiac neural control centers, inducing IST, has not been studied in detail. Therefore, we aimed to characterize functionally and topographically the right atrial neural network.

2 Methods

2.1 Animal Care and Preparation

This investigation was approved by the local authorities (Federal Environmental Agency of North Rhine-Westphalia). Animal care, all experiments and euthanasia were conducted in accordance with the Guide for Care and Use of Laboratory Animals (2008) of the National Institute of Health, USA.

In 16 female sheep (73 ± 11 kg) anesthesia was induced by intramuscular injection of 0.05–0.1 mg/kg of Xylacin (Rompun®) and maintained by continuous administration of pentobarbital (Narcoren®) and fentanyl (Fentanyl-Jansen®) (White and Taylor 2000). Twelve-lead surface ECG, capillary oxygen saturation, and left ventricular pressure were continuously monitored. A bolus of heparin was given (initially 5,000 IE) and repeated as necessary to achieve an activated clotting time 300–400 s. Then vascular sheaths were placed and catheters were advanced under fluoroscopic control into the desired positions. A decapolar electrode-catheter was introduced into the coronary sinus (CS), additional electrodecatheters were placed in the high right atrium (HRA) and the right ventricular apex (RVA). To obtain continuous hemodynamic measurements a pigtail-catheter was placed in the left ventricle (Meyer et al. 2010).

2.2 Mapping

Conventional 3D electro-anatomical mapping of the right atrium (RA) was obtained using the CartoXP® system (Biosense Webster Inc., Waterloo, Belgium). By acquisition of a dense pattern of anatomical points, a representative model of the RA was created. Superior and inferior vena cava

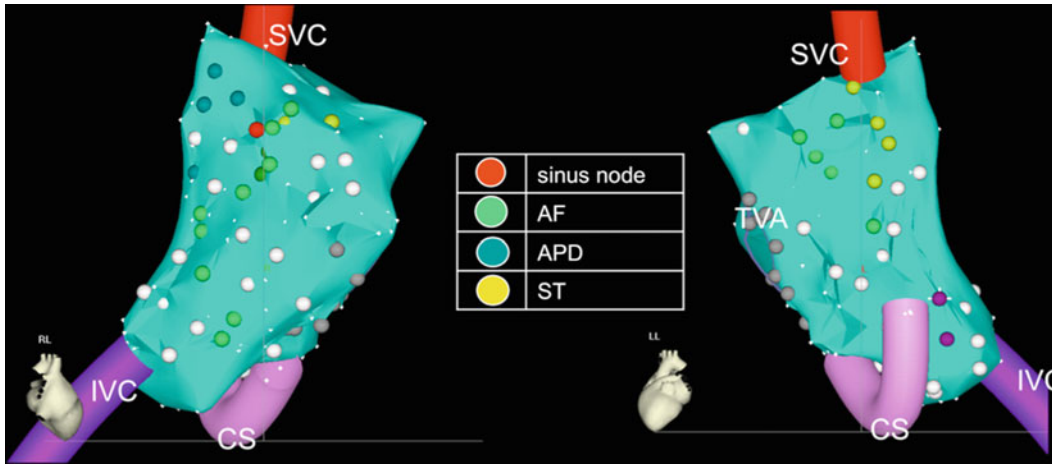

Fig. 38.1 Anatomical map of the right atrium (RA) in the right lateral (RL) and left lateral (LL) projection. The points are color-coded according to certain properties under neurostimulation. The site of earliest activation in the RL projection is marked with a *red* point. Sites where atrial premature depolarizations were localized (APD) were concentrated in the sinus node area, where also the earliest activation during sinus tachycardia (ST) was present. By contrast, high frequency stimulation sites, where atrial fibrillation was induced, were mostly located alongside the course of the crista terminalis (CT) (*white* = no effect, *lilac* = negative dromotrope effect, *grey* = tricuspid anulus, see also legend above; *SVC* vena cava superior, *IVC* vena cava inferior, *TVA* tricuspid valve anulus, *CS* coronary sinus)

(SVC, IVC), the coronary sinus, and the tricuspid valve anulus (TVA) were located and integrated into the map (Knackstedt et al. 2008). In the activation map of the RA, location of the earliest depolarization during sinus rhythm, as measured against the reference electrode located in the CS, was considered to represent the sinus node and marked accordingly in the map.

2.3 Remote Magnetic Navigation

In a subgroup of five animals, remote magnetic navigation (RMN; Niobe®, Stereotaxis Inc., St. Louis, MO) was employed. By applying an adjustable magnetic field, emitted by two compounds of solid state magnets, to a magnetically steerable catheter (Navistar RMT, Biosense Webster) which can be advanced and rotated by a remote-controlled manipulator unit, this system allows for a total remote navigation of the mapping catheter (Pappone et al. 2006). Through integration with the CartoXP® system this technique makes it possible to navigate a compatible catheter inside the preacquired map with high precision and minimal need for fluoroscopic reference.

2.4 Neurostimulation

As has been previously reported, the elements of the ICANS have been selectively stimulated via a transvenous approach (Schauerte et al. 2001). This is facilitated by coupling of high-frequency stimulation trains to a paced rhythm, so that they are delivered during the atrial refractory period (train duration 50 ms, frequency 200 Hz, 5 V, 2 ms pulse duration, delay 20 ms; Grass S-88 Stimulator, Astro-Med, West Warwick, RI), thus preventing unintended myocardial capture (Meyer et al. 2010). Experimental stimulation was performed at multiple sites in the RA. At each site, stimulation voltage was varied stepwise from 10 to 30 V in random order, with a resting interval of minimally 60 s. The exact location of each stimulation-site was marked in the preobtained CartoXP®-map and color-coded according to the observed effects (Fig. 38.1).

PQ, QRS, QT, and QTc were measured during HFS and atrial pacing. For this purpose, the last five beats before the cessation of stimulation were averaged and compared with the intervals with atrial pacing alone before the onset of HFS. If sinus tachycardia ensued, SCL was monitored

for a period of 40 s. Sinus tachycardia was defined as a shortening of the cycle length of at least 10 % with unchanged p-wave morphology and 1:1 atrio-ventricular conduction. This was defined as a sympathetic response. To assess possible dromotropic effects, incremental atrial stimulation via a programmable stimulator (UHS 20, Biotronik SE & Co., Berlin, Germany) with determination of the Wenckebach period was carried out at baseline and during neurostimulation.

In selected locations, when the autonomic response appeared, HFS was repeated after blockade of muscarinic or adrenergic receptors by administration of atropine (3 mg/kg) or propranolol (0.2 mg/kg).

2.5 Histological Workup

In four animals the heart was retained by dissection after the end of the experiment and immediately stored at −80 °C. Further preparation, staining for acetylcholinesterase and histological analysis of the heart was conducted as described previously (Pauza et al. 2002a). The analysis focused on the sinus node area, where most of the effects could be observed during our study.

2.6 Statistical Analysis

Results are presented as mean values ± SD. Neurostimulation effects were analyzed by ANOVA for repeated measurements. A two-sided t-test was used to compare procedural data, $p < 0.05$ was considered significant.

3 Results

3.1 Animal Testing

In 3 of the 16 animals, data acquisition could not be completed due to fatal events during the examination. In two animals refractory ventricular fibrillation was induced by accidental asynchronous stimulation and in one animal unexpected respiratory failure was encountered. Data from these animals were excluded from further analysis.

3.2 Mapping

Creation of the 3D electro-anatomical map was straightforward and comparable to the clinical procedure. In this experimental setting, a large number of annotation points ($n = 227 \pm 89$) was acquired per specimen, to attain the desired accuracy of the right atrial anatomical representation. Difficulties were encountered while mapping the inferoseptal portion of the RA, due to instable catheter position, insufficient wall-contact, and frequent catheter dislocation. Although not being investigated systematically, this resulted in a comparably lower density of mapping points in this area. The problem was partially overcome after an initial learning curve and benefited greatly from the introduction of RMN.

3.3 Location of the Sinus Node

In the tested animals, the site of earliest activation could be found in the cranial part of the posterior or posterolateral wall of the RA, as expected.

3.4 Remote Magnetic Navigation

The use of RMN resulted in a faster progress of the mapping procedure and a reduction in the use of fluoroscopy; yet these effects were not examined systematically. There was no significant difference in the number of acquired points between the conventional mapping group and the RMN group during electroanatomical mapping ($n = 215 \pm 45$ vs. 246 ± 140 points, $p > 0.05$), as well as during neurostimulation ($n = 92 \pm 61$ vs. 103 ± 59 points, $p > 0.05$). The total procedure times were 9.14 h in the conventional mapping group and 7.04 h in the RMN group ($p > 0.05$).

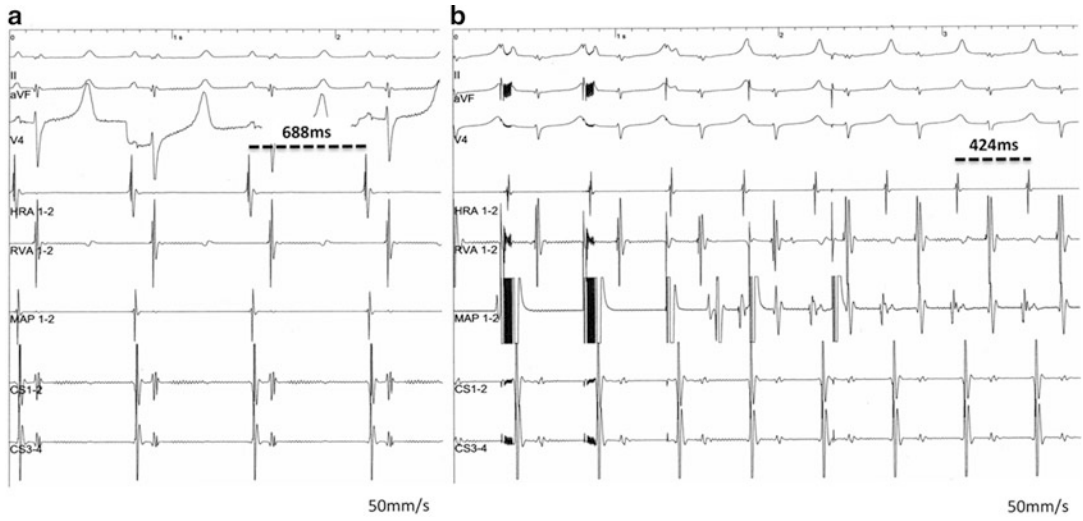

Fig. 38.2 Printout of surface and intracardiac electrocardiograms. (**a**) Readings at baseline. (**b**) Onset of sinus tachycardia after cessation of high frequency stimulation. The initial cycle length of 688 ms is reduced to 424 ms after cessation of high frequency stimulation and pacing. Lead II, aVF, V4, and endocardial electrograms from the high rate atrium (*HRA*), mapping catheter (*MAP*) and coronary sinus (*CS*) are presented

With respect to catheter handling, we can report that most of the manipulations of the mapping catheter could be successfully steered from the control room and only minimal manual support was needed. Almost any desired position in the RA could be reached without lengthy maneuvers. Cather stability was excellent and even in case of dislocation the prior position could be re-assumed easily. This resulted in a more homogenous distribution of mapping points, even in harder to reach areas, and easier execution of the neurostimulation protocol.

3.5 Neurostimulation

Application of the aforementioned protocol for HFS yielded reproducible autonomic responses with characteristic topographic distribution in the RA and could be achieved in all animals. Due to the multiple HFS applications per stimulation site, this took considerably longer than the initial conventional mapping, consequently fewer points were acquired in comparison to the electroanatomic map ($n = 227 \pm 89$ vs. 96 ± 58, $p < 0.05$).

3.6 Sympathetic Effects

Effects mediated by sympathetic elements of the autonomic nervous system were primarily located in the cranial portion of the posterior and posterolateral wall of the RA, with the highest density around the sinus node area (positive chronotropy) as well as along the course of the crista terminalis (positive inotropy). The mean distance of these sites to the point indicating the location of the sinus node was 18 ± 9 mm. At sites were sinus tachycardia could be initiated there was no dose response relationship in respect to stimulation voltage, yet the incidence of points with sinus tachycardia increased with rising stimulation voltage.

3.7 Positive Chronotropy

A rise in heart rate after HFS occurred at 80 sites in 12 animals (Fig. 38.2). If there was any change in p-wave morphology, the site was eliminated from analysis. Maximal reduction of SCL under sinus tachycardia amounted to -20.3 ± 10.1 % ($p < 0.05$). Forty seconds after stimulation, the SCL was still reduced by 5.9 ± 0.1 % ($p < 0.05$).

3.8 Positive Inotropy

In four animals we observed an isolated increase in blood pressure of more than 10 mmHg during HFS. There was no concomitant rise in heart rate during these events, so that an increase of the stroke volume as a result of local excitation of sympathetic fibers has to be suspected.

3.9 Parasympathetic Effects

Effects mediated by cholinergic elements of the ICANS, such as negative dromotropy, were primarily located in the inferior part of the RA, in the area between the ostium of the CS and the junction of the IVC. No lengthening of cycle length indicating sinus bradycardia and/or sinus arrest was observed during HFS within the RA.

3.10 Negative Dromotropy

Negative dromotropic effects could be induced in 12 animals. The effects ranged from a prolongation of the Wenckebach period to 2nd degree AV block with 2:1 or 3:1 conduction and even to a total AV block with resulting asystole. Also there was a prolongation of the PQ interval (mean = 25 ± 22 %, $p = 0.05$). For these effects, a correlation with stimulation voltage was observed. In two animals with pronounced AV blockade under HFS, atropine was administered to abolish cholinergic activation. Subsequently it was not possible to induce any alterations of AV conductance by HFS anymore.

3.11 Proarrhythmic Effects

Sites with induction of APD and AF were located predominantly within a corridor stretching from the roof of the right RA to the ostium of the IVC, corresponding to the course of the crista terminalis. HFS within the atrial refractory period was accompanied by proarrhythmogenic effects in 12 of the 16 sheep, in total AF was inducted at 182 and APD at 130 sites. The position of the CT was verified by referring to the aforementioned anatomical landmarks as well as tactile feedback from the mapping catheter and characteristic electrical properties.

By titrating the stimulation voltage as described above, we observed an increase in the incidence of APD and AF with increasing voltage levels. At sites were sinus tachycardia could be initiated there was no dose response relationship in respect to stimulation voltage. Of interest, in some cases the onset of APD under HFS and the stimulation site were clearly separated. The first depolarization of the APD could be identified in the activation map up to 3 cm apart from the catheter position during stimulation.

3.12 Histological Analysis

Histological work-up in a subgroup of the tested animals ($n = 4$) focused on the region around the sinus node. The general assessment of the procured organs was in accord with prior analysis (Saburkina et al. 2010) and correlated well with the expected distribution being generated from the neuroanatomical map. Special care was taken in the preparation of the sinus node region, which was divided into two perimeters with a radius of 2 and 2–4 cm around the point of earliest activation in the activation map (Fig. 38.3). Quantitative analysis of the neural elements in this area revealed a significantly higher density of ganglia in the inner perimeter compared with the outer perimeter ($n = 19 \pm 5$ vs. 8 ± 2; $p = 0.03$), while the ganglia in the inner perimeter tended to be slightly smaller than those in the outer perimeter (0.15 ± 0.03 vs. 0.17 ± 0.04, $p = 0.0008$). Following the previously described arrangement of the cardiac nervous system (Pauza et al. 2002b), a greater part of the ganglions could be attributed to the dorsal right-atrial subplexus, while there was also substantial input from the ventral right atrial subplexus (Table 38.1).

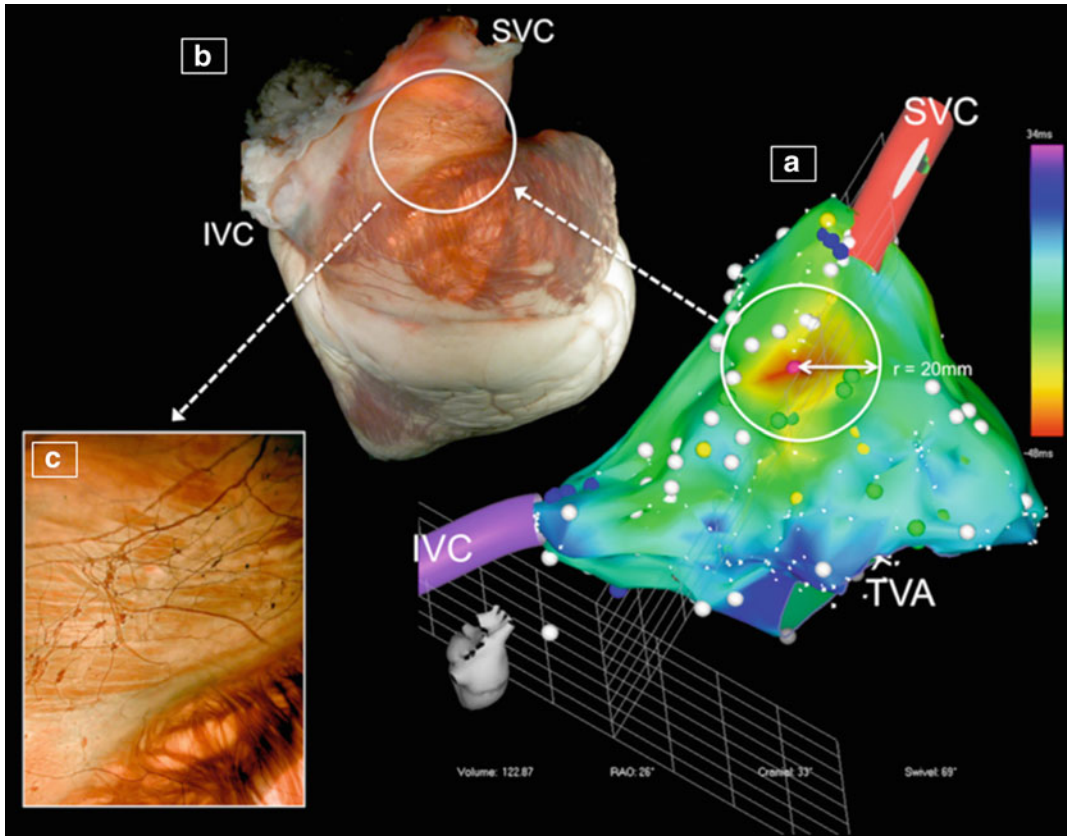

Fig. 38.3 (**a**) Right atrial activation map (RAO projection) demonstrating an area of earliest activation (*arrow*) during sinus rhythm. The *pink dot* next to the *arrow* indicates the catheter-location with the earliest activation, representing the location of the sinus node. The circle around the assumed sinus node location indicates a radius of 20 mm; (**b**) Anatomical preparation of the right RA; (**c**) Magnification of the sinus node area as indicated in the figure. Axons and ganglia dyed for acetylcholinesterase can be seen as *dark lines* and *spots* in the magnification (*SVC* vena cava superior, *IVC* vena cava inferior, *TVA* tricuspid valve anulus, *CS* coronary sinus)

4 Discussion

Our main findings are as follows: (1) By combining standard electroanatomical mapping with HFS during the atrial refractory period we were able to create a 3-dimensional representation of the sympathetic neural network modulating sinus node function; (2) In a perimeter around the sinus node high frequency stimulation predominantly elicited shortening of the cycle length of about 20 %; (3) Histological analysis demonstrated a dense neural network in a radius of 2 cm next to the sinus node area which was identified by electroanatomical mapping.

Our findings indicate a close correlation between the functional and anatomical neural network in the sinus node area. This is of interest since identification of intracardiac neural structures is still challenging. Anatomically and HFS guided neuroablation, as proposed for several heart rate related disorders, has been used with mixed results so far (Pokushalov et al. 2009; Scherlag et al. 2005). This is partly explained by the fact that in the left atrium there is limited accordance between the area of effective HFS application and the anatomical location of intrinsic cardiac neurons (Pokushalov et al. 2009). By contrast, our data suggest a good accordance of the functional and histological evaluation of the elements of the ICANS around the sinus node area.

Table 38.1 Mean number and size of cardiac ganglia in the inner and outer perimeter of the sinus node area

Regions	Neural subplexus	Quantity of hearts	Ganglion number	Mean ganglion size (in mm^2)
Zone around the sinoatrial node (up to 2 cm in radius)				
RSVC	DRA/VRA	n = 4	54 ± 4	0.18 ± 0.06
MRA P	DRA	n = 4	16 ± 2	0.22 ±0.05
SSRAu P	DRA	n = 4	0	0
VSRA P	VRA	n = 4	7 ± 3	0.19 ± 0.07
Average		n = 4	19 ± 5	0.15 ± 0.03
Zone in 2–4 cm radial distance from the sinoatrial node				
MRA D	DRA	n = 4	14 ± 3	0.29 ± 0.07
IRA	DRA	n = 4	7 ± 4	0.09 ± 0.06
SSRAu D	DRA	n = 4	0	0
VSRA D	VRA	n = 4	14 ± 4	0.40 ± 0.06
VIRA	VRA	n = 4	4 ± 2	0.08 ± 0.04
Average		n = 4	8 ± 2	0.17 ± 0.04

Topographical classification according to the classification established by Pauza et al. (2002b)
DRA dorsal right atrial subplexus, *VRA* ventral right atrial subplexus, *IRA* inferior right atrium, *MRA* middle right atrium, *RSVC* root of the superior vena cava, *SSRAu* superior section of the right atrial auricle, *VIRA* ventral inferior right atrium, *VSRA* ventral superior right atrium, *a* "*p*" or "*d*" indicates the proximal resp. the distal part of the according region)

Of interest, HFS around the sinus node did not result in any negative chronotropic effects. This is most probably due to a concentration of sympathetic elements in the sinus node area. As demonstrated by our and other groups in animals and humans, ganglia with predominantly parasympathetic outflow modulating sinus node function are located in the vicinity of the superior caval vein outside the perimeter described above (Hou et al. 2007; Schauerte et al. 2001). Therefore, functional sympathetic and parasympathetic neural structures seem to be selectively attainable in the upper right atrial/superior caval vein area in the structurally healthy sheep heart. Transfer of these findings might be limited by variability of the ICANS between the species (Pauza et al. 2002b). Yet the experience from previous studies suggests a good transferability of topography and stimulation effects between different animal species and humans (Bianchi et al. 2009). If this would hold true for the sinus node area, the presented approach might open up new avenues to control cardiorespiratory autonomic disturbances resulting in sinus tachycardia (Drexel et al. 2013; Tzeng et al. 2007). Transvenous catheter-based ablation approaches targeting sinus node innervation might offer a promising alternative to surgical approaches (Taketani et al. 2007) or direct sinus node modification (Lee et al. 1995). Our findings suggest that partial denervation of sinus node innervation might be feasible, while keeping some distance from the pacemaker tissue itself. Whether ablation of the identified neural structures might abolish sinus tachycardia without modifying sinus node function was beyond the aim of the present study and needs to be demonstrated before any clinical application can be considered.

Although clinical consequences are incompletely understood, it needs to be taken into consideration that we might have induced sinus node reentry tachycardia instead of sinus tachycardia. Whether chronotropic competence and physiological variation of heart rate could be preserved following modification of our identified structures needs to be investigated.

We focused our study on the right atrium, although there is interaction of the autonomic innervation of both atria (Lin et al. 2009). Also cardiac neuromodulation for the treatment of syncope targets structures adjacent to the LA (Pachon et al. 2005). Further studies including the LA should be undertaken to advance our understanding of functional atrial innervation and the interaction between different elements of the ICANS initiating sinus tachycardia.

5 Conclusion

By combining conventional electroanatomical mapping techniques with high frequency stimulation during the atrial refractory period, we here demonstrate functional and topographic concordance of right atrial neural structures inducing sinus tachycardia. Whether this approach could be implemented as a useful tool in the assessment of autonomically mediated disorders needs to be investigated. This might help to advance the clinical application of interventional neuromodulation in patients with inadequate sinus tachycardia.

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

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Occurrence of Disseminated Intravascular Coagulation in 2,3,7,8-Tetrachlorodibenzo-p-Dioxin-Induced Pneumonia in the Rat

39

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Abstract

Intensity of inflammatory reaction in tissue or organ structures depends on the efficiency of homeostatic mechanisms of the organism which limit the extent of this reaction. In studies on the dynamics of inflammatory reaction in induced pneumonia after exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the occurrence of disseminated intravascular coagulation (DIC) has been observed. In this article we evaluated the DIC syndrome in regard to histopathological assessment and laboratory diagnostics of blood. The evaluation indicates that some hematologic indicators (RBC, HCT, and HGB) decreased in the experimental inflammatory reaction, which might be associated with erythrocyte hemolysis in the inflammatory focus and erythrocyte elimination from circulation as a result of DIC. There also were shifts in the number of various leukocyte forms due likely to the accumulation of particular cells in the inflammatory focus. Histopathological assessment of the inflammatory focus revealed the process of hepatization and the occurrence of DIC.

Keywords

Dioxin • Disseminated intravascular coagulation • Fibrinogen • Hematology • Pleuritis • 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

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DOI 10.1007/978-94-007-6627-3_39,

1 Introduction

Dioxins are environmental toxins that can modify the inflammatory response and increase the frequency and intensity of disseminated intravascular coagulation (DIC) (Calkosinski et al. 2011). The DIC syndrome often is a sequel of release of proinflammatory interleukins and damage of the capillary vessel endothelium. This syndrome may be clinically manifest in some areas of the body as bluish spider-like extravasations and by changes in internal organs, namely in lung, kidney, or brain parenchyma. Local inflammatory changes often cause hemolysis, erythrocyte aggregation, adhesion of platelets to detached endothelial cells resulting in microclots and leading to activation of intravascular coagulation (endogenic path) (Couto 2000; Kostro et al. 2000; Jastrzebska 1999). The DIC is a quick and dynamic process. There is an increase in microvessel permeability, with accompanying edema and connective tissue turgidity, which induces fibroblasts to divide and generate morphotic elements of collagen and elastic fibres to demarcate and neutralize toxic inflammatory effects. Slowness of blood flow facilitates diapedesis of leukocyte elements, such as macrophages, and the passage of plasma proteins, such as fibrinogen, into surrounding body tissue. The result is a diagnostically observed syndrome of consumption of blood cells and fibrinogen, an extension of the time bleeding and blood coagulation, and a rise in the degradation products of fibrinogen: D-dimers and plasminogen.

In the present study we investigated the dynamics of inflammatory reaction in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced pneumonia. We focused on the DIC syndrome which developed in response to TCDD, taking into account histopathological changes in lung parenchyma and peripheral blood indices.

2 Methods

2.1 Experimental Animals

The study was approved by a Local Ethics Council for Animal Experiments (permission number: 23/2001). Female rats from *Buffalo* inbreed strain aged 9–11 weeks, weighing 130–150 g were used in for the experiments. The animals were kept under steady conditions of 21–22 °C, 62–62 % humidity in polystyrene cages (60 × 40 × 40 cm), and six animals to a cage, and on a light/dark cycle of 12/12 h. They were fed a standard rodent chow and had water *ad libitum*. The rats were anesthetized with pentobarbital (30 mg/kg, i.p.) after which abdominal cavity was cut open and a canula of 2 mm in diameter was inserted into the aorta to collect blood into standardized hematological and serological tubes (Sarstedt, AG & Co, Nümbrecht, Germany).

The animals were divided into the following groups:

- 30 control rats (C), without any manipulations, in which blood was collected at 120 h of the experiment;
- 60 rats with carrageenan-induced pleuritis, CP Group, which received a single intrapleural injection of 0.15 ml of 1 % carrageenan solution (Sigma-Aldrich, St. Louis, MO) given in the first minute of the experiment. Blood was collected at four time points: 24, 48, 72, and 120 h after carrageenan injection;
- 60 rats injected with a single dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (5 μg/kg, i.m), which then received 0.15 ml of 1 % carrageenan solution intrapleurally on Day 20 after TCDD injection, dioxin + carrageenan group (DCP Group). Here, blood was collected at three time points: 24, 72, and 120 h after carrageenan injection.

2.2 Hematological, Biochemical, and Histopathological Examinations

The following hematological parameters were assessed with an Sysmex XT-1800i apparatus (Sysmex Ltd, Poland): erythrocytes (RBC), hemoglobin (HGB), hematocrit (HCT), platelets (PLT), thrombocrit (PCT), leukocytes (WBC), neutrophils (NE), lymphocytes (LY), monocytes (MO), eosinophils (EO), and basophils (BA).

Biochemical markers in the serum were measured with a Konelab Prime Analyzer 60i (Thermo Electron Corp.; Millcreek Road,

Marietta, OH). Fibrinogen was measured using Fibrinogen-C set (no. 02030110), containing bovine thrombin (no. 0020301110), in an IL ACL Coagulation Analyzer 9000 (Instrumentation Laboratory; Holliston, MA). The calibration and sample procedures were carried out according to CLSI H21-A5 instructions. The C3 and C4 complement components were measured with an immunoturbidimetric method, total protein with Weichselbaum's colorimetric method according to Skeggs and Hochstrasser's modification.

For the histopathological assessment, segments of pleura were collected along with a fragment of lung from the area of carrageenin injection which induced inflammation. Samples were fixed in 10 % formaldehyde, embedded in paraffin, and stained by hematoxylin and eosin.

Data were presented as means ± SD, along with minimum (MIN) and maximum (MAX) values. Data distribution was tested with a Kolmogorov-Smirnov test and the inter-group differences were compared with a *t*-test and Bonferroni correction. $P < 0.05$ was taken as indicating statistical significance. A commercial Statistica 9.0 (StatSoft Ltd.) package was used for data analysis.

3 Results

3.1 Blood Indices in the Course of Experimental Pleuritis

Erythrocytes, Haemoglobin, and **Hematocrit**. In CP Group, the number of RBC decreased between 48 and 120 h of inflammation compared with the control group. In DCP Group, a decrease in RBC was even greater (Table 39.1).

Leukocytes. In CP Group, a significant increase in the number of WBC was observed between 24 and 72 h compared with the control group, abating thereafter. In DCP Group, an increase in WBC was observed after 24 h. The number of WBC returned toward the control level at 72 h and it remained at this level further on up to 120 h (Table 39.2).

Neutrophils. In CP Group, there was a significant increase in a relative value of NE at 24 h compared with the control value; abating thereafter, so that at 120 h the number of NE dropped below the control level. In DCP group, a significant decrease in NE was observed at both 24 and 120 h compared with the control group (Table 39.2).

Lymphocytes. In CP Group, there were more dynamic changes in the number of LY: first a significant decrease in LY at 24 h compared with the control level, then a rebound from 48 to 120 h, ending up with a significant increase above the control at 120 h. In DCP Group, there was an increase in LY compared with the control level (Table 39.2).

Monocytes. In CP Group, the number of MO increased at 48 compared with the control level, flowed by about two-fold decrease below control at 120 h. In DCP Group, a decrease in MO was observed at all time intervals of interest (Table 39.2).

Eosinophils and **basophils**. No appreciable changes in either CP or DCP Group were observed in both EO or BA in the experimental inflammation compared with the control values (Table 39.2).

Platelets and **platelet hematocrit**. In CP Group, appreciable decreases in PLT and PCT were observed at 24 and 72 h compared with the control values, whereas in DCP Group these decreases appeared inappreciable (Table 39.1).

Total protein. TP decreased during the inflammatory process, particularly in CP Group, compared with the control level. The decrease rebounded at 120 h in both CP and DCP Groups (Table 39.1).

Complement. C3 component markedly increased in both CP and DCP Groups at all time intervals measured, compared with the control group. C4 component changes, on the other hand, appeared erratic in CP Group, whereas it also strongly increased in IOD Group (Table 39.1).

Fibrinogen. Concentration of fibrinogen increased in both CP and DCP Groups

Table 39.1 Blood indices at baseline control (C) and then at 24, 48, 72, and 120 h of the experimental inflammatory process

		RBC ($\times 10^6$/μl)	HGB (g/dl)	PLT ($\times 10^3$/μl)	PCT (%)	HCT (%)	TP (g/dl)	C3 (mg/dl)	C4 (mg/dl)	Fibrinogen (g/l)
C	n	14	14	14	14	14	25	20	21	10
	Mean ± SD	8.52 ± 0.26	15.74 ± 0.57	789.4 ± 86.3	0.51 ± 0.06	43.02 ± 1.22	6.29 ± 0.69	1.79 ± 0.82	8.43 ± 2.09	1.09 ± 0.20
	Min–Max	7.90–8.94	14.10–16.10	525–863	0.31–0.56	40.50–44.60	5.30–7.90	0.60–3.20	5.06–12.82	0.91–1.57
CP	n	6	6	6	6	6	31	24	24	7
24 h	Mean ± SD	8.69 ± 0.21	15.78 ± 0.50	642.3 ± 109.3	0.43 ± 0.07	43.48 ± 1.08	5.44 ± 0.96	53.38 ± 19.61	5.41 ± 2.21	1.99 ± 0.44
	Min–Max	8.40–9.04	14.80–16.20	439.0–762.0	0.31–0.51	41.50–44.40	3.10–6.80	26.10–90.30	2.23–10.37	1.58–2.63
		NS	NS	**	*		***	***	***	***
CP	n	6	6	6	6	6	28	19	23	11
48 h	Mean ± SD	7.68 ± 0.22	14.10 ± 0.42	712.5 ± 94.2	0.48 ± 0.06	39.05 ± 1.42	6.01 ± 0.81	68.45 ± 15.84	8.85 ± 2.96	2.18 ± 0.81
	Min–Max	7.44–8.07	13.70–14.90	641.0–890.0	0.45–0.60	38.00–41.70	4.40–8.00	41.60–92.60	3.65–14.15	0.99–3.54
		***	***	NS	NS	***	NS	***	NS	***
CP	n	6	6	6	6	6	31	19	24	6
72 h	Mean ± SD	7.51 ± 0.54	13.60 ± 0.86	600.3 ± 153.0	0.43 ± 0.09	38.18 ± 2.70	5.13 ± 1.27	68.45 ± 15.84	6.00 ± 2.12	1.64 ± 0.22
	Min–Max	6.76–8.33	12.70–15.20	294.0–716.0	0.26–0.48	35.30–43.20	3.50–7.50	41.60–92.60	2.43–9.94	1.29–1.93
		***	***	**	*	***	***	***	***	***
CP	n	9	9	9	9	9	10	7	5	15
120 h	Mean ± SD	7.40 ± 0.58	13.53 ± 0.87	803.3 ± 70.8	0.58 ± 0.05	37.27 ± 2.62	5.96 ± 0.39	35.39 ± 2.79	10.3 ± 1.54	1.39 ± 0.39
	Min–Max	6.01–8.17	13.53–14.70	680.0–890.0	0.50–0.65	31.40–41.50	5.20–6.50	32.30–39.30	8.83–12.19	1.09–2.49
		***	***	NS	**	***	NS	***	NS	*
DCP	n	6	6	6	6	6	5	6	7	6
24 h	Mean ± SD	6.71 ± .35	12.43 ± 0.60	746.83 ± 41.47	0.47 ± 0.03	34.25 ± 1.57	5.96 ± 0.26	50.32 ± 1.71	13.18 ± 2.71	1.52 ± 0.12
	Min–Max	6.37–7.22	11.80–13.30	715.0–815.0	0.44–0.51	32.60–36.20	5.60–6.30	48.60–52.50	10.28–16.90	1.45–1.76
		***	***	NS	NS	***	NS	NS	***	NS
DCP	n	6	6	6	6	6	5	6	7	6
72 h	Mean ± SD	6.52 ± 0.36	12.07 ± 0.62	623.00 ± 74.84	0.40 ± 0.05	33.43 ± 1.66	5.82 ± 0.11	55.70 ± 3.93	25.08 ± 1.32	1.65 ± 0.19
	Min–Max	6.22–7.18	11.30–13.10	526.0–750.0	0.33–0.48	31.10–36.20	5.69–5.96	50.80–59.70	23.24–26.71	1.40–1.90
		**	**	NS	NS	**	NS	*	***	NS
DCP	n	7	7	7	7	7	5	6	7	6
120 h	Mean ± SD	6.92 ± 0.76	12.53 ± 1.09	671.57 ± 192.77	0.46 ± 0.13	34.67 ± 2.98	6.82 ± 0.33	51.47 ± 3.69	34.27 ± 5.26	1.58 ± 0.38
	Min–Max	6.23–8.12	11.20–14.20	269.0–851.0	0.20–0.59	30.90–39.20	6.56–7.38	44.30–55.10	23.41–38.17	1.22–2.20
		NS	NS	NS	*	NS	**	***	***	NS

Values are means ± SD; *n* number of animals

CP carrageenan-induced pleuritis, *DCP* dioxin + carrageenan-induced pleuritis

*0.05 ≥ P > 0.01; **0.01 ≥ P > 0.001; ***0.001 ≥ P; *NS* non-significant

Table 39.2 White blood cell pattern at baseline control (C) and then at 24, 48, 72, and 120 h of the experimental inflammatory process

		WBC	Percentage content of leukocyte forms (%)				
			NE	LY	MO	EO	BA
C	n	14	14	12	12	12	12
	Mean ± SD	5.22 ± 1.33	25.21 ± 6.28	52.36 ± 5.81	12.94 ± 3.61	2.33 ± 1.99	0.41 ± 0.14
	Min–Max	2.72–7.11	13.40–37.70	42.10–59.90	7.80–18.50	0.20–7.00	0.20–0.70
CP	n	6	6	6	6	6	6
24 h	Mean ± SD	7.73 ± 0.67	47.03 ± 17.27	29.62 ± 5.91	14.62 ± 13.72	2.27 ± 1.38	0.30 ± 0.06
	Min–Max	6.71–8.78	27.90–74.40	19.40–37.50	3.40–35.50	0.40–4.50	0.20–0.40
		***	***	***	NS	NS	NS
CP	n	6	6	6	6	6	6
48 h	Mean ± SD	7.75 ± 1.93	24.55 ± 3.28	41.87 ± 5.09	20.50 ± 3.97	4.03 ± 2.14	0.30 ± 0.09
	Min–Max	6.23–11.25	20.50–30.30	34.70–50.20	17.10–27.70	2.50–7.60	0.20–0.40
		**	NS	**	***	NS	NS
CP	n	6	6	6	6	6	6
72 h	Mean ± SD	8.74 ± 2.33	24.12 ± 6.68	48.45 ± 10.54	16.18 ± 9.47	2.82 ± 2.27	0.40 ± 0.14
	Min–Max	7.29–13.02	16.60–33.20	29.40–61.80	8.50–32.70	0.60–6.00	0.20–0.60
		***	NS	NS	NS	NS	NS
CP	n	9	9	9	9	9	9
120 h	Mean ± SD	6.47 ± 2.09	19.36 ± 5.68	73.43 ± 5.83	5.39 ± 3.89	2.28 ± 1.00	0.54 ± 0.68
	Min–Max	3.6–10.3	10.60–29.80	64.00–84.70	1.90–12.60	0.50–3.70	0.00–1.90
		NS	*	***	***	NS	NS
DCP	n	6	6	6	6	6	6
24 h	Mean ± SD	8.03 ± 1.82	12.48 ± 11.67	74.40 ± 13.52	4.60 ± 2.85	1.32 ± 1.65	0.88 ± 1.03
	Min–Max	4.70–10.00	3.90–31.40	60.60–93.40	1.60–9.60	0.20–4.40	0.00–2.90
		NS	**	***	NS	NS	NS
DCP	n	6	6	6	6	6	6
72 h	Mean ± SD	6.32 ± 0.39	28.70 ± 3.70	60.68 ± 5.72	5.35 ± 1.80	2.90 ± 0.40	0.60 ± 0.43
	Min–Max	6.10–7.10	24.40–35.00	52.70–68.20	3.50–8.10	2.60–3.70	0.10–1.40
		*	NS	*	*	NS	NS
DCP	n	7	7	7	7	7	7
120 h	Mean ± SD	6.06 ± 2.65	12.99 ± 9.28	80.07 ± 10.27	4.67 ± 3.86	1.06 ± 1.26	1.21 ± 1.23
	Min–Max	1.80–8.70	4.60–26.70	67.50–92.00	1.60–12.80	0.10–3.50	0.00–2.80
		NS	NS	NS	NS	*	NS

Values are means ± SD; *n* number of animals
CP carrageenan-induced pleuritis, *DCP* dioxin + carrageenan-induced pleuritis
*0.05 ≥ P > 0.01; **0.01 ≥ P > 0.001; ***0.001 ≥ P; *NS* non-significant

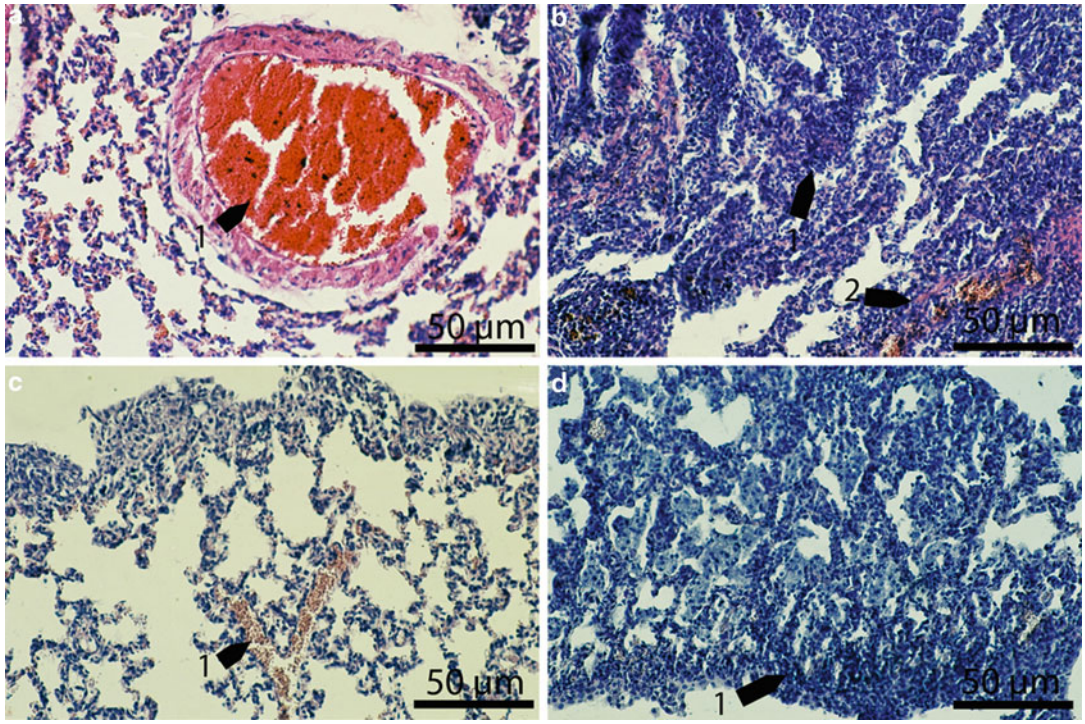

Fig. 39.1 Pleuritis and DIC syndrome in lungs. (**a**) DIC syndrome in lungs: *1* – clot in a blood vessel; (**b**) *1* – thickening of the interalveolar septa with accumulation of leukocytes, *2* – stasis of blood in the vessels; (**c**) *1* – stasis of blood in the vessels and clot formation; (**d**) *1* – accumulation of leukocytes, red and rust hepatization of lungs

throughout the inflammatory process compared with the control level, although the increased failed to be statistically significant in DCP Group (Table 39.1).

3.2 Histopathology of Pleuritis and Pneumonia

In CP Group, in samples collected from the inflammatory focus at 24 h from the onset of reaction, proliferation of pleural epithelial cells (mesothelium) was observed, along with the accompanying serous exudates mixed with sparse inflammatory cells, like neutrophilic granulocytes, macrophages, and lymphocytes; the typical symptoms of pleuritis. In pulmonary parenchyma, there were foci of catarrhal or fibrinous inflammation with hyperemia and edema, or reddish areas of hepatization. There also was DIC observed (Fig. 39.1). A clinical symptom of DIC is tail livedo, which histopathologically manifest as necrosis of tail skin and panniculus, with subsequent hyaline degeneration and tissue calcification. The walls of blood vessels also underwent necrosis as a result of the accumulation of fibrin formed in DIC (Fig. 39.2). In samples collected at 48 and 72 h, the inflammatory reaction intensified and the number of lymphocytes and macrophages in pulmonary parenchyma increased. At 96 h, proliferation of lymphatic follicles at the edge of inflammatory foci was observed. At 120 h, the pleura thickened in comparison with the earlier time groups, which manifest as multiple mesothelial cell layers (Fig. 39.3).

In DCP Group, in samples collected from the inflammatory focus at 72 and 120 h from the onset of reaction, there were visible signs of lymphohistiocytic bronchitis and bronchiolitis. Inflammation of the alveolar walls with bronchial tissue, lymphoid follicles, and glandular proliferation was also observed (Fig. 39.4).

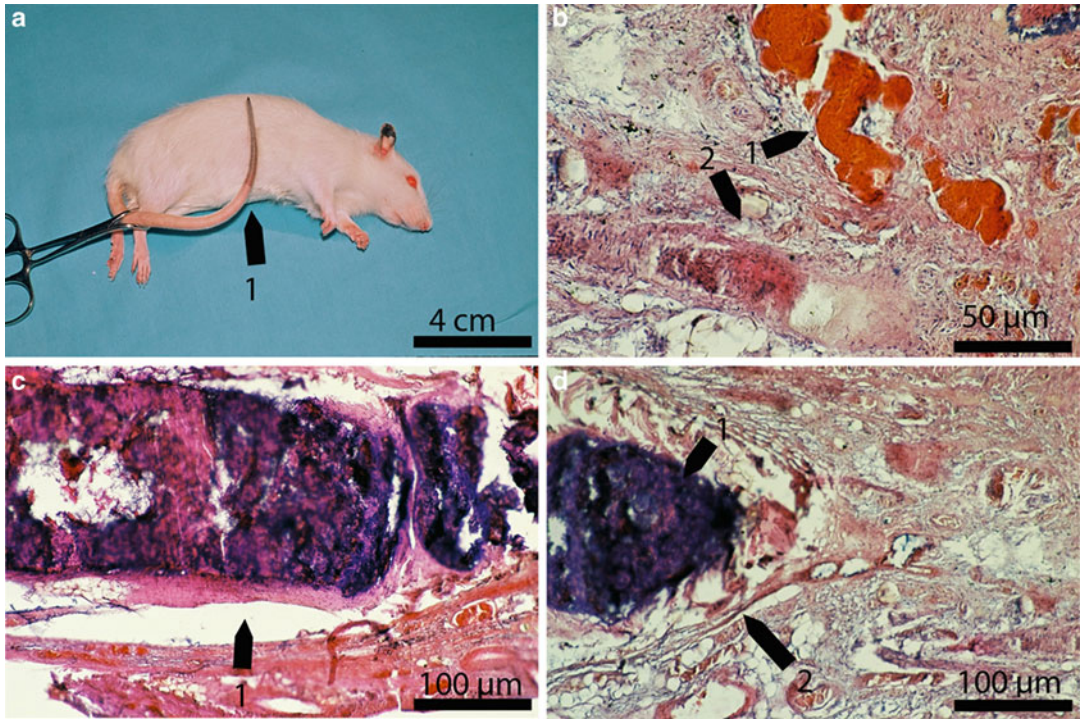

Fig. 39.2 DIC syndrome, necrosis, and focal calcification in the rat tail. (**a**) *1* – Tail livedo; (**b**) *1* – extravascular blood clots, *2* – necrosis of blood vessel wall; (**c**) *1* – coagulation necrosis in panniculus with subsequent hyaline degeneration and tissue calcification; (**d**) *1* – necrosis, *2* – tissue calcification

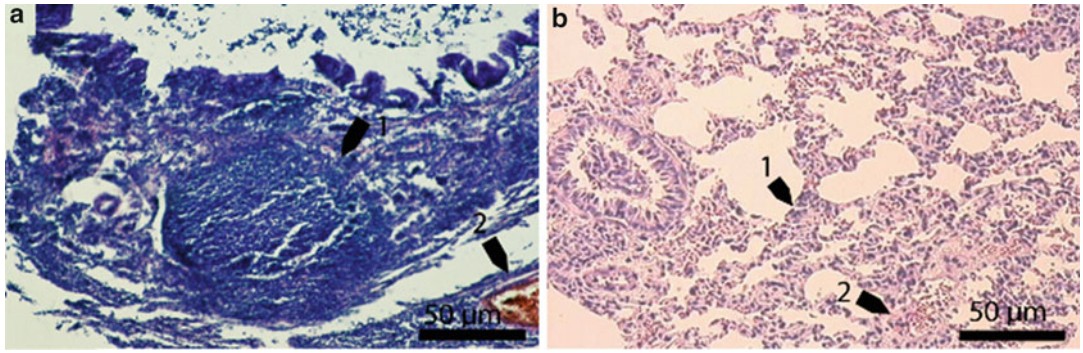

Fig. 39.3 Catarrhal inflammation. (**a**) *1* – proliferation of lymphatic follicles in lungs, *2* – blood vessels filled with erythrocytes; (**b**) 120th h of inflammation: *1* – multiplied mesothelium cells arranged in a few layers, thickened interalveolar septa; *2* – numerous clots in blood vessels

4 Discussion

In the carrageenan-induced inflammation, used as a reference in this study, decreases in the number of erythrocytes, hematocrit, and in the amount of hemoglobin were observed between 48 and 120 h. The decreases can be explained by the waste of some erythrocytes in the inflammatory focus in the lungs and in the distal tail segment (Wasiutynski 1978). Other authors have observed a decrease in hematocrit already

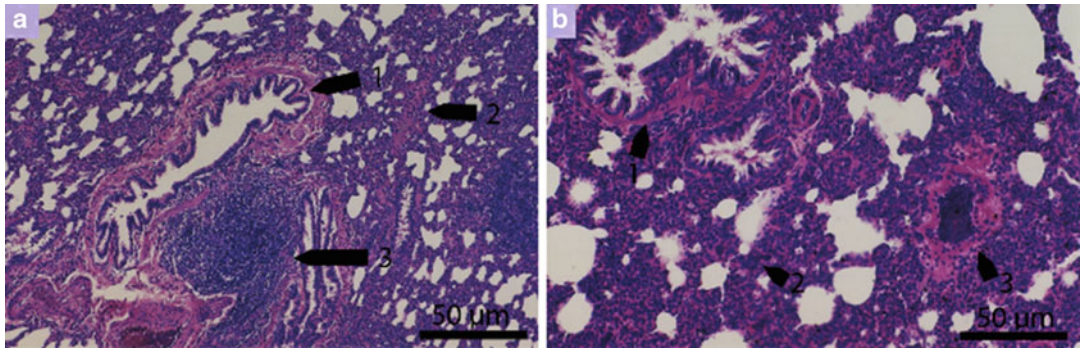

Fig. 39.4 Pleuritis and DIC syndrome in lungs after TCDD administration. (**a**) 72th h of inflammation. *1* – swollen blood vessel wall, *2* – thickened interalveolar septa, *3* – inflammation of alveolar walls and bronchial tissue, proliferation of lymphoid follicles and glands; (**b**) 120th h of inflammation. *1* – lymphohistiocytic bronchitis and bronchiolitis; *2* – accumulation of leukocytes; *3* – numerous clots in blood vessels

after 24 h from the commencement of inflammation, with its subsequent increase later on (Weimer and Benjamin 1965). Moreover, in the inflammatory focus hemolysis of erythrocytes might occur due to changes in physicochemical properties, such as decreased pH, increased partial pressure of CO_2, activation of the complement system, which all lessen erythrocyte resistance (Marianska 2002).

In the present study, at this early stage of inflammation, we observed an increase in the number of leukocytes, particularly neutrophils, due likely to their being attracted by chemotactic factors, including the C5 component of complement (Wysocki 1990; Zeman et al. 1996). A subsequent decease in the number of neutrophils with the passage of time is explicable by their accumulation in the inflammatory focus, followed by marginalization through adherence to the vessels walls and then migration into tissue (Majewska 2003). According to other studies, neutrophils are capable of three-fold faster overpass from the vascular lumen to the inflammatory focus than monocytes (Zeman et al. 1996). Neutrophils accumulating at the inflammatory site release a platelet activating factor (PAF) that affects the aggregation of platelets (Wysocki 1990), the process that, in turn, activates granulocytes and creates a favorable environment for the deposition of fibrin (Dabrowska 1997). These processes are in line with a significant decrease in the number of platelets in response to inflammation, observed in the present study as well as in other studies (Pinkowski and Paradowski 1996).

It may be posited that the above-mentioned hematological alterations correspond in fact to the DIC which develops as a result of inflammation (Sawicka 2002). This assumption finds support in the histopathological signs of DIC observed in inflammatory specimens in the present study. Furthermore, the assumption is supported by enhanced consumption of antithrombin III due to inflammatory reactions found in biochemical studies (Buller and Cate 1989).

In the histopathology of the present control, carrageenan-induced inflammation we observed signs of developing pleuritis and focal pneumonia with accompanying exudates. Moreover, in the inflammatory focus, accumulation of phagocytic elements was found, such as neutrophils, macrophages, and lymphocytes whose proportional participation depended on the duration of inflammatory process. Rapid accumulation of neutrophils in inflammatory foci, connected with ischemia, may take place already after 20 min (Zeman al. 1996). In the present study, with increasing time of inflammation, fibrin deposits and red hepatization appeared. The processes observed can be explained by a decrease in the number of erythrocytes that were swept away into the fibrinogen net of the inflammatory focus (Calkosinski et al. 2003; Laurent et al.

1988). The number of platelets also decreased, a phenomenon observed during the occurrence of DIC by others as well (Weimer and Benjamin 1965). The DIC in the distal segment of the rat's tail, along with carrageenin-induced pleuritis, we observed is rather difficult to interpret. It might have to do with a weak microcirculation, overreactivity of vessels, or greater concentration of proinflammatory interleukins, especially TNF, in the tail region (Calkosinski et al. 2009; Calkosinski et al. 2011).

In the present study, administration of TCDD affected erythropoiesis and reduced the numbers of erythrocytes, hemoglobin, and hematocrit; the results in line with those reported in other studies (Ivens et al. 1993, 1992; Zinkl et al. 1973). There was no significant difference in the total number of leukocytes in the DCP group compared to control. However, there were appreciable changes in the white blood cell pattern. This was manifest in the predominance of lymphocytes over neutrophils and in the reduced number of monocytes and eosinophils. The literature data suggest that such changes may have to do with age of experimental animals (Knutson and Poland 1980). Application of large doses of tocopherol may counteract the occurrence experimental pleuritis in rats (Calkosinski et al. 2011).

5 Conclusion

In an experimentally induced inflammatory reaction of the pleura, decreases in erythrocytes, hematocrit, and hemoglobin were observed; likely being associated with erythrocyte hemolysis in the inflammatory focus and with the occurrence of disseminated intravascular coagulation. Erythropenia might also have to do with disturbed erythropoiesis, as other blood indices such as MCV, MCH, and MCHC were inappreciably affected. Leukocyte forms shifted from peripheral blood into the inflammatory focus, where they accumulated as confirmed by the histopathological picture. The histopathological examination revealed hepatization in the vicinity of a lung lobe confined into the inflammatory reaction and the occurrence of disseminated intravascular coagulation after 24 h from inflammation induction, which provided a rational explanation for decreases in the peripheral blood indices. In the course of pleuritis, the most intensive changes in biochemical indicators occurred between 48 and 72 h of inflammation and consisted of a decline in total protein concentration and increases in complement components and fibrinogen. Addition a dioxin derivative to carrageenan-induced pleuritis resulted in longer term downward changes in red blood cell indices and a more strongly expressed signs of disseminated intravascular coagulation

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

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Implementation of Non-invasive Methods in the Diagnosis of Diisocyanate-Induced Asthma

40

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Abstract

Diisocyanate-induced asthma is difficult to diagnose since the immunopathological mechanisms and exposure determinants at the workplace are not well defined. The aim of this study was to evaluate the non-invasive methods of nasal lavage fluid (NALF) and induced sputum (IS) to enhance the diagnostic efficiency. Sixty-three diisocyanate-exposed workers with work-related shortness of breath underwent a standardized 4-steps-1-day-whole body exposure test with diisocyanates used at work up to 30 ppb. NALF and IS were collected before, 0.5, and 19 h after the end of exposure. Cellular composition and soluble inflammatory biomarkers were studied in the samples. In addition, ten controls with bronchial hyperresponsiveness, but without prior occupational diisocyanate exposure, were also examined. Twelve out of the 63 subjects (19 %) showed a significant asthmatic reaction (pulmonary responders) after challenge (FEV_1 decrease >20 %). NALF samples did not demonstrate significant effects either on cellular composition or on mediator concentrations in the responders, non-responders, or controls at any time point. In contrast, in the IS samples of the pulmonary responders collected 19 h after challenge, the percentage of eosinophils was higher ($p = 0.001$) compared with baseline before challenge. Eosinophils were also increased 30 min and 19 h after challenge in IS samples of the responders compared with the non-responders or controls. In addition, 19 h after challenge the eosinophilic cationic protein (ECP) concentration was significantly higher in the responders than non-responders ($p < 0.04$) or controls ($p < 0.002$). In conclusion, positive asthmatic reactions to diisocyanates are accompanied by an influx of

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_40,

eosinophils into lower airways. Analysis of induced sputum should be implemented in the diagnostic procedure of diisocyanate-related airway diseases.

Keywords
Diisocyanate • Eosinophilic inflammation • Induced sputum • Nasal lavage fluid • Occupational asthma

1 Introduction

Isocyanates and their products are important industrial chemicals related to occupational diseases. They are characterized by one or more isocyanate groups (–N = C = O) and exhibited strong chemical reactivity (Fisseler-Eckhoff et al. 2011; Wisnewski et al. 2006; Raulf-Heimsoth and Baur 1998). The most relevant commercial products are the monoisocyanate methyl isocyanate (MIC) and the diisocyanates 1,6-hexamethylene diisocyanate (HDI), 2,4- and 2,6-toluene diisocyanate (TDI), 1,5-naphthylene diisocyanate (NDI) and 4,4′-diphenylmethane diisocyanate (MDI) as well as oligomers and prepolymers of these isocyanates. Diisocyanates are widely used in the production of polyurethane foam, adhesives, plastic packaging material, laminates, polyurethane paints, and coatings. The compounds also are present in households, e.g. in paints or construction foam. Exposure to diisocyanates occurs mainly by inhalation and depends on the concentration and temperature during the manufacturing process. During recent years diisocyanates have been recognized as one of the main causes of occupational asthma (OA) induced by low-molecular-weight chemicals in Western countries (Paris et al. 2012; Vandenplas et al. 2011), but especially in the Asian-Pacific area (Wisnewski et al. 2006). The underlying mechanisms of diisocyanate-induced OA remain still unclear (Fisseler-Eckhoff et al. 2011; Raulf-Heimsoth and Baur 1998). At high concentrations, diisocyanates can have direct toxic effects on mucous membranes and at low concentrations they act as sensitizing agents after binding to different body proteins. *In vitro* studies supported a role of cell-mediated immune responses. The unequivocal diagnosis of diisocyanate asthma remains quite a challenge. Clinical history, questionnaires and physiological studies are frequently not definitive. Immunological tests have shown variable correlations with disease. Modern approaches in future development of laboratory tests are necessary (Palikhe et al. 2011). The prevalence of diisocyanate specific IgG- and IgE-antibodies among individuals with diisocyanate asthma is variable and not closely associated with the disease (Pronk et al. 2007). Specific inhalation challenge is considered the "gold standard" for diagnosis.

The aim of our study was to implement the non-invasive methods of the collection and analysis of nasal lavage fluid (NALF) and induced sputum (IS) in the diagnosis of diisocyanate-induced asthma.

2 Methods

2.1 Subjects and Protocol

The study was approved by the Ethics Committee of the Ruhr University Bochum and was conducted in accordance with the Declaration of Helsinki of the World Medical Association. All study participants gave written informed consent to the study protocol.

Sixty-three diisocyanate-exposed workers (85.7 % males, 34.9 % non-smokers and 23.8 % smokers; 27 % atopics) with work-related respiratory symptoms referred for a medical opinion by the accident insurance were examined at our institute. Ten subjects (30 % males, 50 % non-smokers, 30 % smokers, 70 % atopics) with bronchial hyperresponsiveness but without any prior occupational diisocyanate exposure were examined

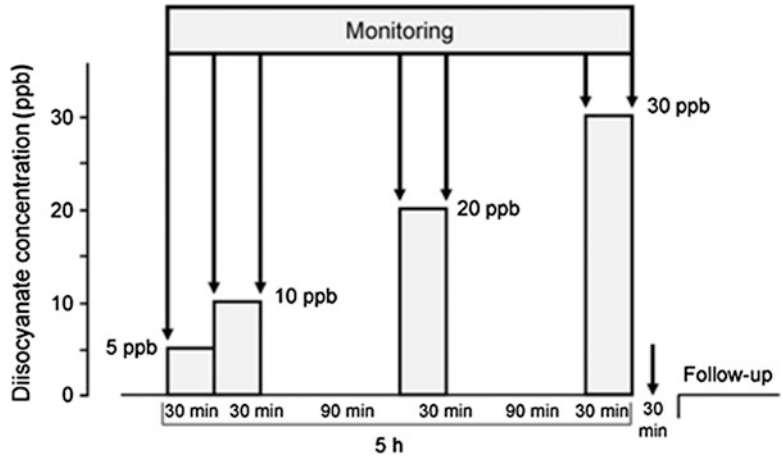

Fig. 40.1 Work-related, standardized 4-steps-1-day diisocyanate-exposure test. Duration of exposure: 2 h (4 × 30 min), duration of the test: 5 h. NALF and sputum collection: before, 30 min, and 19 h after exposure

in the same way (controls). All subjects underwent a work-related challenge test with the dominant diisocyanate used at work. They were not exposed to diisocyanates for the last 5 days before the challenge with MDI (in 40 cases), TDI (in 6 cases), HDI (in 18 cases) and NDI (in 2 cases). The reference group was challenged with both TDI and MDI.

A standardized 4-step-1-day diisocyanate-exposure test was used. The diisocyanate atmospheres were generated in the air tide glass chamber as described previously (Marczynski et al. 2005; Merget et al. 2002). The concentration of diisocyanate vapor in the chamber was measured continuously with a calibrated tape monitor system. All participants in our study were continuously exposed to diisocyanates in four different concentrations, 5 ppb for 30 min, 10 ppb for 30, 90 min break; 20 ppb for 30, 90 min break; and 30 ppb for 30 min. The total diisocyanate exposure time was 2 h and the time from the beginning to the end of challenge was 5 h (Merget et al. 2004). Nasal lavage fluid and induced sputum were collected before, 30 min, and 19 h after exposure (Fig. 40.1).

2.2 Collection and Analysis of Nasal Lavage Fluid and Induced Sputum

Before, 30 min, and 19 h after challenge, nasal lavages were collected and analyzed as described earlier (Raulf-Heimsoth et al. 2011, 2000). Briefly, a syringe with 7 ml 0.9 % saline to which a nasal olive was added for close nostril fitting, was inserted into the left nasal cavity. The saline was passed slowly into the nasal cavity and back into the syringe five times. The recovered volume was recorded and stored at 4 °C.

After centrifugation, aliquots of the cell-free supernatants were stored at −80°C under argon protection until further analysis of soluble markers. The cell pellets were suspended in phosphate-buffered saline and the total cell number was determined in a Neubauer cell chamber.

IS was collected by inhalation of isotonic saline aerosol, generated by an ultrasonic nebulizer for 10 min. The subjects were motivated to actively cough, clear their throat, and expectorate sputum. The sputum processing started with selecting all viscid portions from the expectorated samples for minimizing contamination with saliva. The volume of IS was determined and an equal volume of 0.1 % sputulysin (dithiothreitol) was added. The samples were mixed gently by vortex mixer and incubated for 30 min at 37 °C. After centrifugation, the cell-free supernatants were aliquoted, stored at −80°C under argon protection until further analysis. The cell pellets were resuspended and the total cell number was determined.

For differential cell counts of NALF or sputum cells, slides were prepared by cytospin (Cytospin 2, Shandon Corp., Pittsburgh, PA) and stained with May-Grünwald-Giemsa. Three independent observers counted 200 cells on each slide by light microscopy. The results were

expressed as the percentage of a total cell number and the absolute number of the cell population (without correction for squamous cells).

The inflammatory mediators were determined in the thawed cell-free supernatants of NALF and IS samples. All samples underwent only a single freeze-thaw cycle. The following soluble markers were measured: interleukins IL-5 and IL-8, NO derivatives, total protein, and eosinophilic cationic protein (ECP; using the ImmunoCAP system from Phadia, Uppsala, Sweden). IL-5 and IL-8 were measured with the OptEIA™ ELISAs (BD Biosciences Pharmingen, Heidelberg, Germany) in a standard range of 2–500 pg/mL for IL-5 and 3–200 pg/mL for IL-8.

The amount of NO derivatives was measured by a colorimetric assay kit from Alexis™ (Cayman Chemicals; Grünberg, Germany) determining the total nitrate/nitrite concentration. The sensitivity of the assay was 5 µM and the standard range was between 0 and 35 µM. Total protein content was determined according to the method of Bradford with bovine serum albumin as standard solution (range 10–100 µg/mL) (Bradford 1976).

2.3 Statistical Analysis

Data were analysed with a GraphPad Prism vr. 5.01 for Windows (GraphPad Software, San Diego, CA). Values distribution was assessed using the D'Agostino & Pearson omnibus normality test. Values below the limit of quantification (LOQ) were set 2/3 of the LOQ. Comparisons of unpaired data were performed with a Mann-Whitney U test. For statistical analysis we used the adjusted mean ± SD. A-two-sided significance level of 0.05 was chosen for all tests.

3 Results

Twelve out of the 63 subjects (19 %) showed an FEV_1 decrease >20 % after the challenge. Seven of these responders reacted to MDI (7/40; 18 %), two responded to TDI (2/6; 33 %) and one to HDI (1/18; 6 %). Two challenge tests were performed with NDI and both patients showed a positive response. Seven out of the 12 responders showed an early (immediate) type reaction (four of them challenged with MDI, one with HDI, and two with NDI) and three showed a dual reaction profile (all were challenged with MDI). Delayed type reactions were detected in two responders, both challenged with TDI. Fifty one diisocyanate-exposed subjects did not respond during or after the diisocyanate challenge test and were denominated as the non-responders. The characteristics of the three groups are summarized in Table 40.1. The age was similar in all groups, as was the smoking status. Forty two percent of the responders and only 23 % of the non-responders were atopics. According to our criteria of involving persons with bronchial hyperresponsiveness into the reference group, most of them were atopics (70 %). Median duration of occupational exposure to diisocyanate was different between the responders and non-responders; 8 and 20 years, respectively.

Table 40.1 Characteristics of tested persons

	Responders	Non-responders	Controls
Numbers (n)	(12)	(51)	(10)
Gender (males)	10 (83 %)	44 (86 %)	3 (30 %)
Age (years; median)	37	42	35
Smoking status	4 NS	18 NS	5 NS
	3 S	12 S	3 S
	4 Ex	10 Ex	2 Ex
	1 n.d.	11 n.d.	–
Atopics	5 (42 %)	12 (24 %)	7 (70 %)
Years of occupationally exposed workers (median)	8	20	–

NS non-smoker, *S* smoker, *Ex* ex-smoker, *n.d.* not documented

The cellular and mediator profiles of NALF and IS samples of 12 responders were compared with those of non-responders and 10 reference persons. Analysis of NALF, independent of the clinical reaction profile during and after the diisocyanate challenge test, showed no significant differences regarding either the cellular profile (especially eosinophil and neutrophil count) or the concentration of IL-4, IL-8, ECP, NO, or total protein (data not shown).

In contrast to NALF, in the IS samples of responders, the percentage of eosinophils significantly increased 19 h after challenge ($p = 0.001$) compared with baseline. In IS samples collected 30 min and 19 h after challenge, the percentages of eosinophils in the responders were significantly higher than in the non-responders and controls (both $p < 0.001$). No significant differences in the percentages of eosinophils were detected in IS samples before, 30 min, and 19 h after challenge between the non-responders and controls (Figs. 40.2 and 40.3). In 86 % of the IS samples of responders collected 19 h after challenge, more than 100 eosinophils/mL were detectable, whereas only in 11 % of the IS samples of non-responders and in no IS sample of controls this was the case. In addition, also the percentage of neutrophils increased after challenge in the responders (before: 6 %, 30 min: 12 %, 19 h: 20 % (median values)) and non-responders (before: 9 %, 30 min: 9 %, 19 h: 12 % (median values)), without reaching the significance level. No significant differences in the percentages of neutrophils were measured at any time point between the three groups.

The same analysis was performed for the ECP concentrations in IS: an increase of ECP concentrations after challenge in the responder group without similar increase in the non-responders and controls was detected. Nineteen hours after challenge significant differences in the level of ECP between the responder and non-responder groups ($p < 0.04$) and the responders and controls ($p < 0.002$) were measured. Thirty minutes after challenge no significant differences were observed between the three groups (Fig. 40.4).

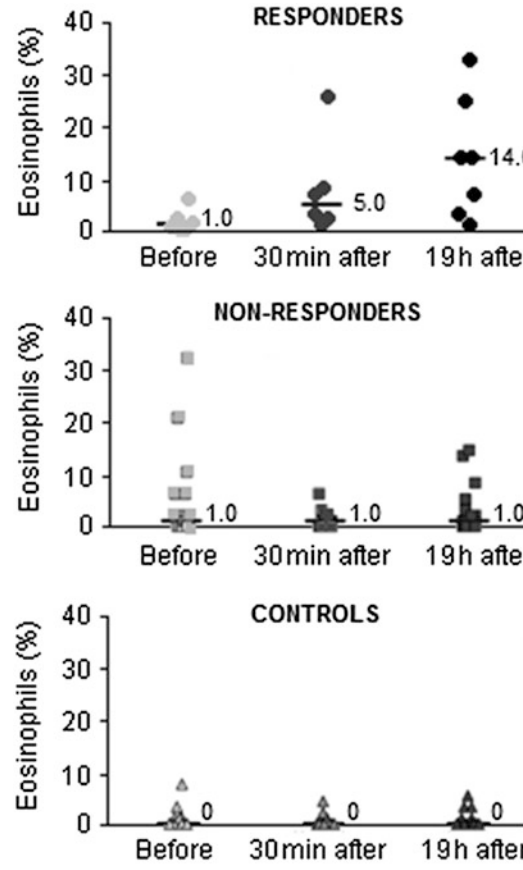

Fig. 40.2 **Percentage of eosinophils** in induced sputum samples of responders, non-responders, and controls before, 30 min, and 19 h after diisocyanate challenges

Concentrations of IL-5 slightly increased 19 h after diisocyanate challenge in the responder group, but no significant differences between IL-5 levels in the non-responders and controls were detectable (Fig. 40.5). In 67 % of the IS samples of responders, the IL-5 levels were above 15 pg/mL (median: 19.6 pg/mL). 19 h after challenge, this was the case in only 20 % of non-responders (median: 5.6 pg/mL) and in 20 % of the controls (median: 6.7 pg/mL). Concentrations of IL-8 showed high inter-individual variability (range between <3.0 and 84,248.0 pg/mL), but in the IS samples of the responder group the IL-8 levels (median: 3,123.0 pg/mL) 19 h after challenge were higher than in the non-responders (median:

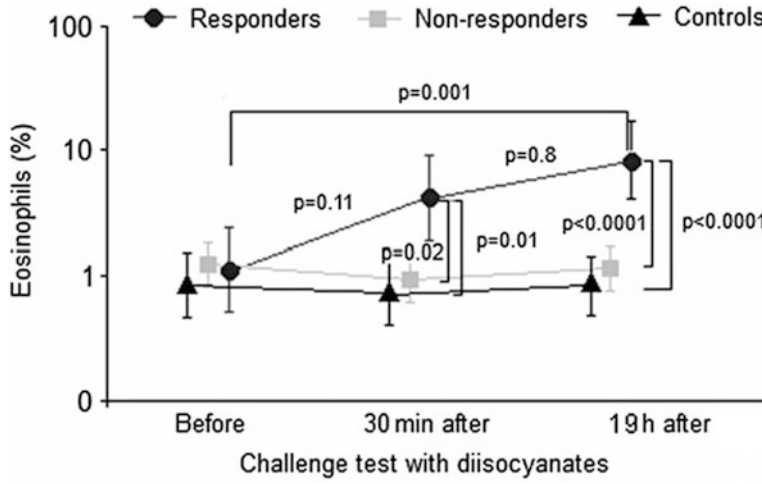

Fig. 40.3 Comparison of eosinophils (%) in induced sputum samples obtained from the subjects in the three groups (responders, non-responders, and controls) before, 30 min, and 19 h after diisocyanate challenges

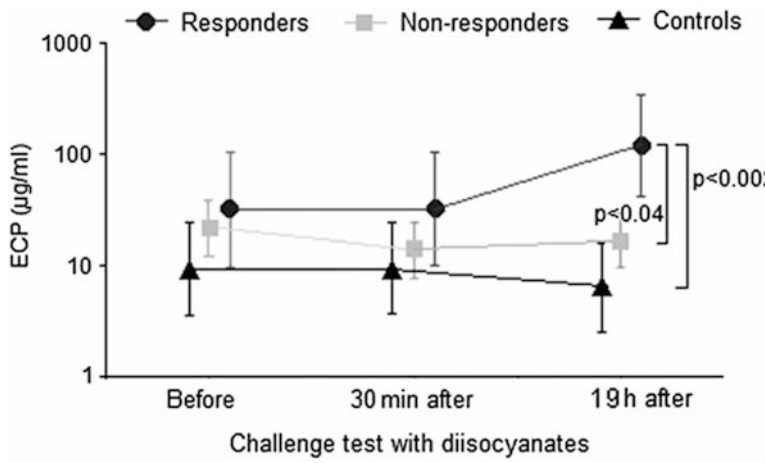

Fig. 40.4 Comparison of eosinophilic cationic protein (ECP)-concentrations in induced sputum samples obtained from the subjects in the three groups (responders, non-responders, and controls) before, 30 min, and 19 h after diisocyanate challenges

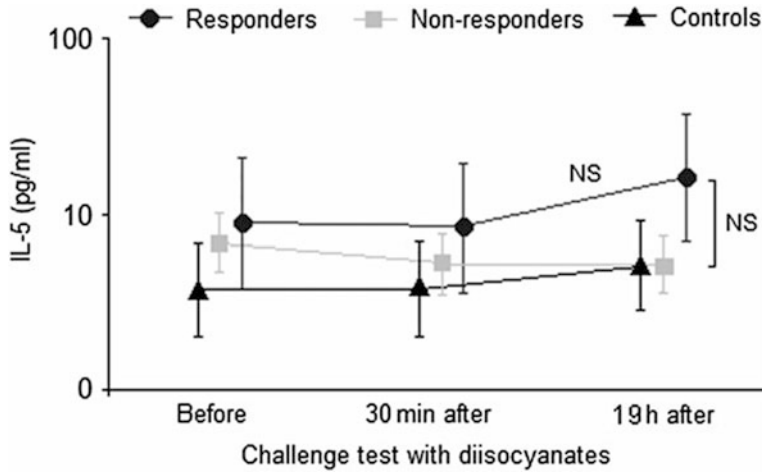

Fig. 40.5 Comparison of IL-5 concentrations in induced sputum samples obtained from the subjects in the three groups (responders, non-responders, and controls) before, 30 min, and 19 h after diisocyanate challenges

1,366.0 pg/mL) and controls (648.0 pg/mL), but without statistical significance.

4 Discussion

Collection of NALF and IS and analysis of specific cellular and soluble biomarkers are useful non-invasive tools to assess upper and lower airway inflammation. The major result presented in this study is the detection of eosinophilic inflammation in the lower airways in a group of workers responding with a FEV_1-decrease >20.0 % to the diisocyanate challenge. In the IS samples, the percentages of eosinophils as well as ECP- and IL-5 concentrations of subjects with diisocyanate-induced positive reactions were increased 30 min and/or 19 h after challenge compared to baseline. Comparison between the IS profile of responders, non-responders, and controls indicated that the increase of eosinophils after challenge is the most sensitive sputum parameter for the responder allocation. Although our study is limited in the number of responders, the increase of eosinophils in the IS samples of responders was nearly independent of the type of asthmatic reaction (early, late, or dual) and independent of the inducing diisocyanate. In the case of occupational asthma induced by high molecular weight substances, the majority of subjects developed an eosinophilic airway inflammation after exposure, e.g., during a specific inhalation challenge with the relevant culprit. In the EAACI Task Force Consensus Paper (Quirce et al. 2010) the authors concluded that an increase in sputum eosinophil counts greater than 3 % after specific inhalation challenge often precedes the occurrence of functional changes on subsequent exposure. In 85 % of the IS obtained from the responders after diisocyanate challenge in our study eosinophil counts greater than 3 % were detected. In contrast, in only 11 % of the non-responders and 13 % of the controls the eosinophil percentages were greater than 3 % after challenge. The characteristics of eosinophilic inflammation were also supported by an increase of soluble factors like IL-5, a protein produced by several different cells including CD4+ T cells, mast cells and eosinophils, is involved in the development, survival, and activation of eosinophils and the eosinophil granule-derived cationic protein (ECP). Therefore, it is possible to conclude that positive reactions to diisocyanates are accompanied by an influx of eosinophils in the lower airways, documented by induced sputum analysis. Lemière et al. (2002) also showed in their study with 12 diisocyanate-exposed patients and IS analysis an acute inflammatory response, but with only a moderate increase of eosinophils and a dominant influx of neutrophils instead. In our study, the percentage of neutrophils also increased, but without significant differences between the responders and non-responders. In addition, differences in the study design (lower diisocyanate concentration during challenge test and induction of sputum with hypertonic saline solution) by Lemière et al. (2002) may explain the differences in the outcome compared to our study. In contrast to our study, Park et al. (1999) found that activated neutrophils may contribute to bronchoconstriction induced by TDI which may be associated with IL-8 release. In that study, the authors focused on neutrophil activation with the parameter chemotaxis, myeloperoxidase, and IL-8 analyzed in IS at the last time point of 7 h after challenge. The differences (neutrophilic vs. eosinophilic inflammation) may be caused by kinetics of cell influx: starting with a quick, but more or less unspecific, increase of neutrophils after challenge and only in responders, the eosinophils increased at later time points (e.g., the next day, 19 h after challenge) as a sign of (sub)-chronic inflammation.

In contrast to high molecular weight occupational allergens like latex (Raulf-Heimsoth et al. 2000), diisocyanates did not induce detectable cellular or mediator changes in the upper airways, documented by analysis of NALF. NALF has been previously described as an accepted non-invasive method to objectively measure nasal inflammation (Quirce et al. 2010). Therefore, these results indicate that although the nasal cavity is the primary route of entry for inhaled air and the first region of the respiratory tract to be in contact with the diisocyanate, the upper airways are not significantly affected by diisocyanate.

In conclusion, positive asthmatic reactions to diisocyanates are accompanied by an influx of eosinophils to the lower airways. Therefore, in the diagnostic procedure of diisocyanate-related airway diseases the analysis of induced sputum with focus on eosinophilic inflammatory markers should be implemented.

Acknowledgements This study is part of a doctor thesis of R. Liebig and part of the IPA-projects All/Imm-D and Med-D funded by the DGUV (German Social Accident Insurance).

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

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Evaluation of a 4-Steps-1-Day Whole Body Challenge Protocol for the Diagnosis of Occupational Asthma due to Diisocyanates

41

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Abstract

Inhalative challenges are important in the diagnosis of occupational asthma due to diisocyanates. As existing protocols are time-consuming and costly, it was the aim of this study to develop a short duration whole body exposure protocol. Ninety three subjects with suspected occupational diisocyanate-induced asthma and verified current or previous occupational exposure to diisocyanates and ten control subjects without diisocyanate exposure but with bronchial hyperresponsiveness were investigated. After baseline examination on the first day, subjects underwent a standardized whole body multiple-steps-1-day challenge with exposures of up to four times 30 min to concentrations of 5, 10, 20, and 30 ppb of the dominant diisocyanate used at work on the second day. Common spirometric and body plethysmographic parameters were used as positivity criteria. Overall, 14 subjects demonstrated a positive diisocyanate challenge, 19 were considered doubtful, and 60 were negative. All controls had negative challenges. Positive reactions occurred during the challenge ($n = 10$) or during follow-up ($n = 4$). Eight subjects showed >40 % fall of FEV_1. These severe reactions occurred after 5 ppb ($n = 2$) or 10 ppb ($n = 3$), while isolated late reactions after 2 h of follow-up were not observed. Multivariate analysis showed an association between a positive challenge and both the degree of previous occupational exposure and the presence of baseline bronchial hyperresponsiveness. In summary, the proposed 4-steps-1-day diisocyanate challenge protocol induced pronounced bronchial reactions in a small number of subjects. As these reactions were more likely to occur after low concentrations, it is recommended to shift the initial concentration/dose step to lower exposures.

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_41,

Keywords
Asthma • Bronchial hyperresponsiveness • Challenge • Diagnosis • Diisocyanates • Occupational

1 Introduction

Inhalation challenges are considered an important tool to confirm the diagnosis of occupational asthma (OA) (Vandenplas et al. 2006). While in most forms of OA the need for inhalative challenges may be questioned due to the relatively high prediction of sensitization in subjects with work-related symptoms (van Kampen et al. 2008), the demonstration of sensitization in diisocyanate asthma is rare. The sensitivity of *in vitro* assays for the detection of diisocyanate-specific IgE is too low to serve as a reliable diagnostic marker (Wisnewski 2007). Thus, exposure protocols of diisocyanate challenges are needed that yield a high diagnostic efficiency, without posing a danger to the subjects' health.

Diisocyanate challenges have been performed as whole body exposures and by a closed-circuit apparatus. To our knowledge, the closed-circuit method was used exclusively in Canada (Malo et al. 1999; Vandenplas et al. 1992a), a comparison between both methods showed minor differences with respect to challenge results. Whole body exposures are performed with different protocols in many countries; most studies used stepwise challenges with 2–4 dose steps (Vandenplas et al. 1992a; Vogelmeier et al. 1991; Smith et al. 1980). Whereas exposures were performed with the most common diisocyanates (isophorone diisocyanate (IPDI), toluene diisocyanate (TDI), diphenylmethane-4,4′-diisocyanate (MDI), naphthylene-1,5-diisocyanate (NDI), and 1,6-hexamethylene diisocyanate (HDI)), protocols differ with respect to exposure and test duration, and minimal and maximal diisocyanate concentrations. Exposure monitoring is almost exclusively performed using tape monitor systems; with minimal concentrations of mostly 5 ppb and maximal concentrations of mostly 20 ppb showing little differences between studies (Karol et al. 1994; Vandenplas et al. 1993; Banks et al. 1989; Mapp et al. 1986a, b; Malo et al. 1983; O'Brien et al. 1979). Only few researchers used higher concentrations, such as 30 ppb (Smith et al. 1980), 40 ppb (Paggiaro et al. 1987), or 250 ppb (Paggiaro et al. 1986). While the total exposure duration of stepwise tests showed little variation with a range of approximately 30–120 min (Malo et al. 1999; Vandenplas et al. 1996; Moscato et al. 1993; Tossin et al. 1989; Vogelmeier et al. 1991; Paggiaro et al. 1987; Mapp et al. 1986a, b; O'Brien et al. 1979), the most important differences between study protocols were the total test duration, i.e., most researchers included long periods without exposure to yield total test duration of 2 days (Vandenplas et al. 1992a, 1996; Malo et al. 1983; Smith et al. 1980) to 3 days (Malo et al. 1999; Kopferschmitt-Kubler et al. 1998).

To our knowledge, there is only one single multiple-steps-1-day protocol reported in the literature, with concentrations between 5 and 20 ppb, but the authors did not describe the concentrations of the steps in detail (Vogelmeier et al. 1991).

2 Methods

2.1 Subjects

The study was approved by the Ethics Committee of the Ruhr University and all subjects gave informed consent. Between 2001 and 2008, a total of 122 subjects with current or previous occupational exposure to diisocyanates were investigated in our institute for suspected occupational asthma (OA) or hypersensitivity pneumonitis (HP) due to diisocyanates. Fourteen subjects were not challenged because of baseline

airways obstruction, one subject did not allow the challenge, one asymptomatic subject had transient acute symptoms after accidental high exposure, and in two subjects the diagnosis of HP due to diisocyanates was made by a typical history and increased specific IgG antibodies to diisocyanates. Eleven subjects did not complete the challenge protocol due to burning eyes (n = 1) or hyperventilation (n = 10). In total, 93 subjects were included in the final analysis. In addition, ten control subjects with mild to very mild asthma and bronchial hyperresponsiveness without exposure to diisocyanates were challenged with TDI and MDI (Table 41.1). The challenges in exposed subjects were performed for a medical opinion.

2.2 Clinical Investigations

A complete examination was performed on three consecutive days. During the first day, baseline parameters were recorded, including methacholine testing and repeated lung function measurements to document a stable respiratory status. On the second day, the diisocyanate challenge was performed, and follow-up measurements were terminated on the third day. Medication was stopped since the first day. Anamnestic data were recorded with a self-administered questionnaire that was corrected during a physician's interview. Detailed information about job titles and exposure conditions were obtained from both the patients and the files of the technical services of the accident insurance, which were available for each patient. Both the quality of the dominant diisocyanate exposure and a quantitative expert assessment of inhalative exposure to diisocyanate vapors or aerosols (ratings 1-low, 2-intermediate, and 3-high) were recorded by last author (RM). Environmental monitoring at workplaces was available in 20 cases, the results were considered in the expert judgement. Personal data (age, gender, and smoking habits), current or previous work-related symptoms (shortness of breath, wheezing, recurrent cough, runny or itching nose, sneezing, burning or itching eyes, and skin complaints of face, forearms or hands) and current asthma medication (bronchodilators and inhalative steroids) and the following information about the course of symptoms were gathered: overall duration of occupational exposure to diisocyanates, period from the beginning of exposure to the onset of symptoms (latency), period of symptomatic exposure, and period from the end of exposure to diagnosis. After a physical examination, standard laboratory tests, electrocardiograms and chest radiographs were performed. Total IgE and specific IgE antibodies to HDI, TDI and MDI were measured in the patients' sera by ImmunoCAP (Phadia, Uppsala, Sweden) according to the manufacturer's recommendations. Skin prick tests (SPT) were performed with a panel of 20 environmental allergens (various manufacturers). Atopy was defined by a wheal diameter of at least 3 mm greater than the saline control (in the presence of a positive histamine control). Body plethysmography and spirometry were performed with MasterScreen (CareFusion, Würzburg, Germany), and specific airway resistance (sRt) as well as forced expiratory volume in 1 s (FEV_1) in percent of predicted (American Thoracic Society 1995) were recorded. Methacholine testing was done with a reservoir method (American Thoracic Society 2000). A positive reaction was assumed if FEV_1 decreased by at least 20 % with the highest dose. As this test is comparable to the ATS dosimeter test, this definition includes subjects that would be considered borderline according to ATS (Baur et al. 1998).

2.3 Diisocyanate Challenges

Diisocyanate challenges were performed with the predominant diisocyanate used at work. Challenges were performed as a whole body exposure as described recently (Marczynski et al. 2003). Briefly, subjects were exposed to MDI (technical grade; n = 51), TDI (n = 6), HDI (n = 30), NDI (n = 4) or IPDI (n = 2). NDI was obtained from Bayer, Leverkusen (Germany), the other diisocyanates from Merck, Darmstadt (Germany). During the challenges subjects were continuously exposed to diisocyanates at four different concentrations

Table 41.1 Personal data, symptoms, and clinical data according to challenge results

	Exposed subjects					
	Challenge result					
	Positive (n = 14)	Doubtful (n = 19)	Negative (n = 60)	Total (n = 93)	Controls (n = 10)	P*
Age; year (median, range)	43 (20–60)	48 (29–63)	43 (20–72)	44 (20–72)	39 (27–52)	0.9
Male gender; n (%)	10 (71)	17 (90)	55 (92)	82 (88)	2 (20)	0.06
Current smoker; n (%)	6 (43)	2 (11)	20 (33)	28 (30)	3 (30)	0.6
Current asthma medication; n (%)	12 (86)	12 (63)	33 (55)	57 (61)	1 (10)	0.03
Job titles; n (%)						0.4
Spray painter	1 (7)	3 (17)	19 (32)	23 (25)	0 (0)	0.06
Foam/casting	3 (21)	3 (16)	6 (10)	12 (13)	0 (0)	0.2
Foundry	1 (7)	2 (11)	1 (2)	4 (4)	0 (0)	0.3
Isocyanate production	2 (14)	4 (21)	7 (12)	13 (14)	0 (0)	0.8
Miner	0 (0)	1 (5)	1 (2)	2 (2)	0 (0)	0.6
Others	7 (50)	6 (32)	26 (43)	39 (42)	10 (100)	0.7
Duration of exposure; mo (median, range)	43 (7–348)	202 (6–459)	120 (0–492)	136 (0–492)	–	0.3
Beginning of exposure to onset of symptoms; months (median, range)	13 (1–255)	168 (1–276)	69 (1–396)	73 (1–396)	–	0.3
Symptomatic exposure; months (median, range)	27 (3–180)	72 (1–291)	34 (1–360)	34 (1–360)	–	0.8
End of exposure to diagnosis; months (median, range)	9 (0–22)	17 (1–220)	8 (0–156)	9 (0–220)	–	0.9
Work-related symptoms						
Airways; n (%)	14 (100)	16 (84)	56 (93)	86 (93)	–	0.3
Nose or eyes; n (%)	5 (36)	5 (26)	24 (41)	34 (37)	–	0.7
Skin; n (%)	3 (23)	2 (11)	9 (15)	14 (15)	–	0.5
FEV_1; %pred (median, range)	91.1 (61.8–136.6)	92.1 (53.7–127.2)	94.4 (59.9–130.0)	92.7 (53.7–136.6)	101.5 (72–126)	0.5
sRt; (median. range)	1.2 (0.6–2.7)	0.9 (05.–2.9)	1.0 (0.5–3.0)	1.0 (0.5–3.0)	1.3 (0.6–2.1)	0.03
Hyperresponsiveness; n (%)	12 (86)	6 (32)	32 (53)	50 (54)	10 (100)	0.03
Atopy; n (%)[a]	2 (15)	3 (16)	23 (39)	28 (31)	–	0.11
Total IgE; kU/L (median. range)	61 (4–833)	19 (4–284)	53 (1–949)	41 (1–949)	30 (11–355)	0.6

Percentages refer to the number of tests performed ([a]2 missings)
*Challenge positives were compared with challenge negatives

for 30 min each: 5, 10, 20, and 30 ppb within 1 day. After the 20 and 30 ppb exposures, a pause of 90 min was made to detect initial late reactions. Thus, the total exposure period was 2 h, the total challenge duration 5 h. Follow-up measurements were performed immediately after the last exposure step, and 30 min, 2 h, 4 h, and about 20 h (next day) afterwards. Peak expiratory flow (PEF) measurements were performed by the subjects between 4 and 20 h follow-up measurements at intervals of 1 h, with the exception of sleeping periods. Body plethysmography and spirometry were performed before and after each step. The diisocyanate atmospheres were generated in an air-tight glass chamber. The concentrations of diisocyanate vapor in the chamber were measured continuously with a calibrated tape monitor system (Toxic Gas

Monitor Series 7100; Zellweger Analytics, Inc., Lincolnshire, USA). The test was considered positive (end-of-test-criterion) if the following three criteria were fulfilled: (1) decrease of FEV_1 of at least 20 %, (2) increase of sRt of at least 100 %, and (3) increase of sRt to at least 2 kPa · s. A test was considered doubtful if the first criterion, but not the second and third, was fulfilled, or if any combination of two of the three criteria was measured. A challenge was defined negative if all three criteria were not fulfilled. Early reactions were defined as positive reactions during the challenge (including the measurement after the last exposure step of 30 ppb); late reactions fulfilled the positivity criterion afterwards. For safety reasons, the subjects spent the night after the challenge in an adjacent hospital, irrespective of the challenge result.

2.4 Statistical Analysis

Characteristics and clinical data of the subjects with positive and negative diisocyanate challenges were compared by a Chi^2 test for categorical variables and a Mann–Whitney *U* test or *t*-Test as appropriate. An extended Mantel-Haenszel Chi^2 test for linear trend was used for the comparison of challenge positive and negative subjects with respect to the three exposure categories. Multivariate analysis was performed with logistic regression analysis. Goodness of fit was assessed by a Hosmer and Lemeshow test. For multivariate analysis only positive and negative challenges were included. $P < 0.05$ was considered statistically significant. Data were analyzed with SPSS version 19, IBM, Ehningen, Germany.

3 Results

The patients were predominantly males (88 %), the median age was 44 (range 20–72) years (Table 41.1). Overall, they had near-to-normal lung function with a mean FEV_1 of 93 % predicted, FEV_1 variability during the first day was below ±10 %. About one third of subjects were current smokers and atopics. Sixty one percent of the subjects were prescribed asthma medication. Job titles were diverse with some preponderance of spray painting, foam/casting and diisocyanate production. Twenty-one subjects (23 %) were still exposed at their workplace during the last month. Work-related airways symptoms were more often reported than symptoms of nose, eyes, or skin. A higher number of control subjects than patients were considered hyperresponsive to methacholine (100 vs. 54 %, respectively), but fewer controls were on current asthma medication (10 vs. 61 %, respectively; Table 41.1).

Overall, 14 subjects (15 %) demonstrated a positive challenge, 19 (20 %) were considered doubtful, and 60 (65 %) were negative. Challenge positive subjects were more likely to take asthma medication than challenge negatives (86 vs. 55 %, respectively) and to experience bronchial hyperresponsiveness (86 vs. 53 %, respectively). They also had somewhat shorter exposure duration and latency (beginning of exposure to onset of symptoms), although this was not statistically significant. There were no further relevant differences between the groups with respect to personal characteristics, exposure data, symptoms, spirometry, or total IgE (Table 41.1). None of the subjects showed sensitization to any diisocyanate.

Of the subjects with positive challenges, ten reactions were positive with MDI (20 % of MDI challenges), three with NDI (75 %) and one with HDI (3 %). None of the ten control subjects showed a positive reaction (data not shown). Five subjects preferred to terminate the positive reaction by a bronchodilator (additional two subjects with negative challenges took medication). By definition, challenge negatives did not show an effect on lung function, but in doubtful cases there was some variation of FEV_1 (Fig. 41.1), but not in sRt (Fig. 41.2). The subjects showed positive reactions (both FEV_1 and sRt-criterion fulfilled) after all challenge steps: 5 ppb (n = 3), 10 ppb (n = 3), 20 ppb (n = 2), and 30 ppb (n = 2), or follow-up measurements after 30 min (n = 1), 2 h (n = 2), or 4 h (n = 1). PEF recordings did not show any positive reactions in subjects with doubtful or negative challenges (data

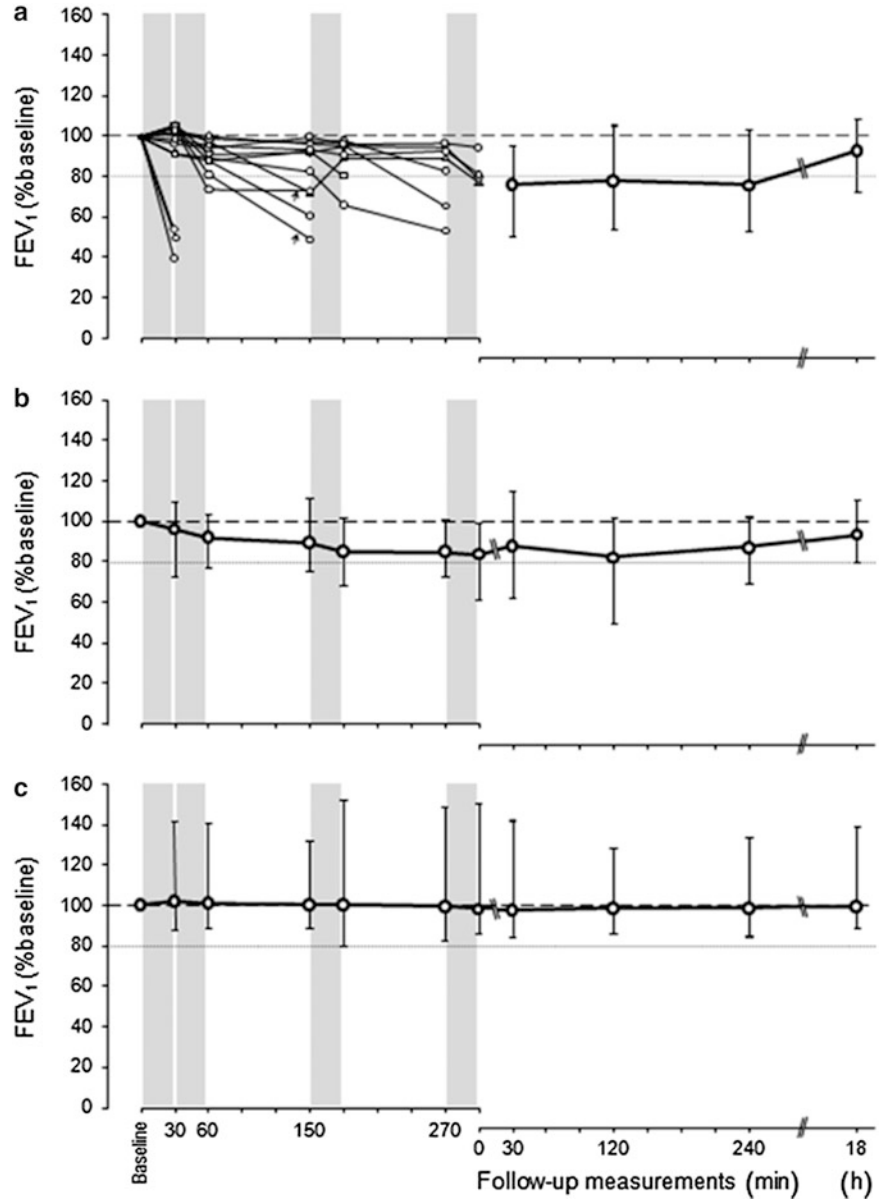

Fig. 41.1 **Time response curves of FEV_1** during challenge and follow-up periods. For challenge positives (**a**), the individual curves are shown for the challenge period, whereas medians and ranges are given for the follow-up period. For subjects with doubtful (**b**) or negative (**c**) challenges, medians and ranges are given for both challenge and follow-up periods. Exposures (4 × 30 min) are represented by grey columns. The two arrow heads in *Panel a* indicate two subjects who showed a significant decrease in FEV_1, but no increase in sRt after the exposure step. In these subjects the test was terminated after a significant reaction was obtained after a pause of 90 min

not shown). However, there were discrepancies between FEV_1 and sRt with respect to the time of significant reactions (data not shown in detail). Of the four subjects with reactions in the follow-up period, three subjects were near-positive during the challenge period. The subject without any reaction during the challenge period became positive at 2 h of follow-up.

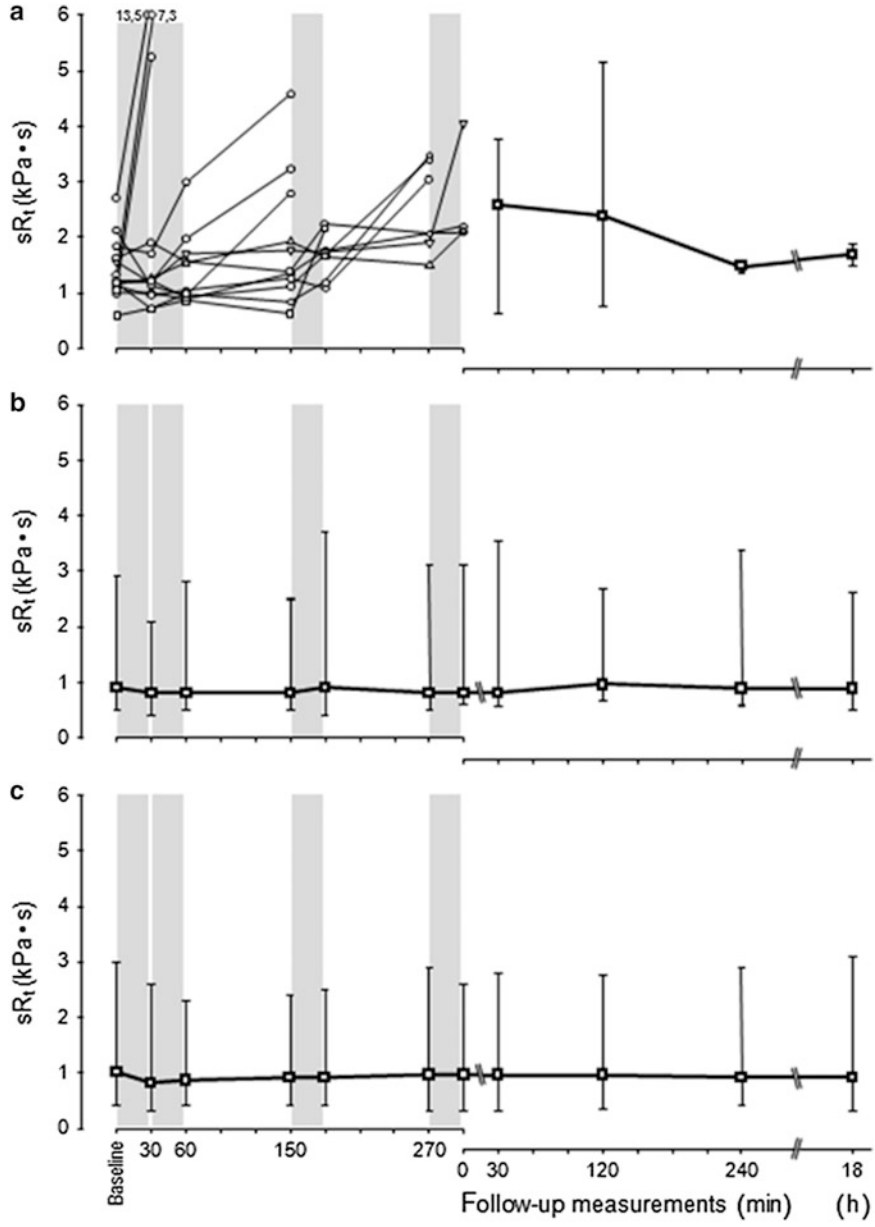

Fig. 41.2 **Time response curves of specific airway resistance** (**sRt**) during challenge and follow-up periods. For challenge positives (**a**), the individual curves are shown, whereas for subjects with doubtful (**b**) or negative (**c**) challenges, medians and ranges are given. The exposure periods (4 × 30 min) are represented by *grey* columns

Severity of reactions during the challenge period and afterward was comparable. The maximal fall of FEV_1 was 60.7 % from baseline during the challenge period, while it was 65.1 % during the follow-up (Fig. 41.3). Eight subjects showed a more than 40 % fall of FEV_1 at any time during the study, five of these inhaled a bronchodilator after the positive reaction. These reactions occurred after 5 ppb (n = 2) or 10 ppb (n = 3). Three subjects needed a second and one subject a third

bronchodilation. In the morning after the challenge day, two subjects showed a FEV_1 decrease >20 % (24 and 28 %) from baseline (both subjects inhaled bronchodilators earlier). These subjects were again treated with a short acting beta-agonist and airway obstruction was fully reversible. They were given topical steroids afterward for a few days and told to contact their general practitioners if symptoms should persist.

The degree of exposure to diisocyanates during employment, as assessed by expert rating, was associated with positive challenges, although a prediction was not possible in each case. Low exposure assignment did not exclude a positive challenge (Table 41.2). This finding was corroborated by multivariate analysis, which in addition showed an association between a positive challenge and bronchial hyperresponsiveness (Table 41.3).

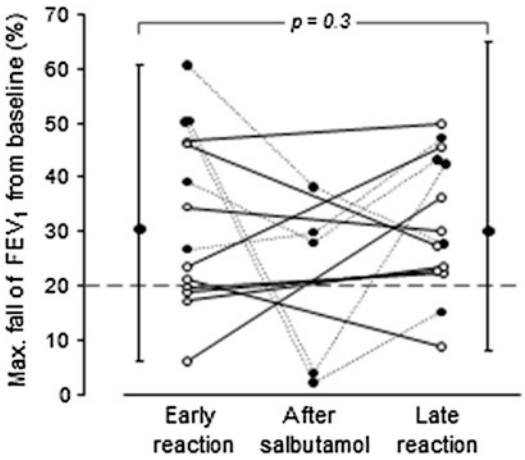

Fig. 41.3 **Maximal fall of FEV_1** during the challenge (*early reaction*) and during follow-up (*late reaction*) in 14 challenge positives. In addition, medians and ranges are presented. Five subjects received a short acting beta-agonist during the challenge (*black dots* and *dashed lines*)

4 Discussion

We describe a highly selected group of subjects with previous or current exposure to diisocyanates and work-related respiratory symptoms. Interestingly, specific IgE antibodies to diisocyanates in serum were not detected in any subject, although we used a common commercially available test. We cannot explain this unexpected finding, but it showed convincingly that positive challenges with diisocyanates may occur in subjects without obvious sensitization. Total IgE was not different between challenge positive and negative subjects, indicating no IgE-dependent mechanism.

To our knowledge there are no studies in the literature that compare different protocols of challenge tests for the diagnosis of occupational asthma with respect to test duration or minimal and maximal doses during challenge steps. Evaluations of existing exposure protocols are descriptive as direct comparisons cannot be performed due to ethical reasons, thus the conclusions that can be drawn from such studies remain limited. This is also true for the present study which intended to shorten the test duration without loss of safety for the patients. However, there are several important findings that may help to establish optimized challenge protocols.

As diisocyanate challenges have the distinctive feature of frequent late reactions, immediate reactions, e.g., after the final dose step cannot be distinguished from late reactions to previous

Table 41.2 Association of challenge results with exposure intensity according to expert ratings

	Challenge results				
Exposure group	Positive (n = 14)	Doubtful (n = 18)	Negative (n = 61)	Total (n = 93)	OR (95 % CI)[a] pos. vs. neg.
1 = low	6	13	45	65	1
2 = intermediate	4	4	14	21	2.1 (0.5–8.7)
3 = high	4	2	1	7	30 (2.9–315)

[a]Extended Mantel-Haenszel chi square test for linear trend

Table 41.3 Odds ratios for a positive challenge from multivariate analysis

	n	OR	95 % CI	p
Exposure degree				
Small (Ref.)	51	1.00		
Medium	18	1.52	0.29–7.93	0.6198
High	5	23.00	1.05–503.03	0.0464*
Latency (month)		0.98	0.92–1.04	0.5025
Symptomatic exposure duration (month)		1.00	0.99–1.01	0.9820
Hyperresponsiveness				
No (Ref.)	30	1.00		
Yes	44	10.19	1.04–99.85	0.0462*
Atopy				
No (Ref.)	47	1.00		
Yes	25	0.27	0.04–1.74	0.1690
Smoking				
Never (Ref.)	31	1.00		
Current	26	0.71	0.12–4.11	0.6994
Ex	17	0.16	0.01–1.94	0.1481
Age (year)		0.96	0.89–1.03	0.2699

Hosmer and Lemeshow Goodness of fit test: $p = 0.5001$. *significant association with responder status

dose steps in 1-day protocols. Thus, maximal doses with optimal sensitivity and specificity cannot be derived precisely from our study. In a limited number of controls we had no false positives, thus specificity seems high up to 30-min exposures of 30 ppb. Concerning sensitivity, an important number of subjects, i.e., 6 of the 14 responders (43 %) demonstrated a positive reaction after the final concentration of 30 ppb. Although some of them might have become positive also with a concentration of 20 ppb at follow-up, a higher concentration than 20 ppb for 30 min may be warranted. Vandenplas et al. (1993) observed that neither the concentration nor the duration of exposure *per se* is the main determinant of the occurrence of an asthmatic reaction to diisocyanates, but the dose that is the product of both factors. As the generation of higher exposure concentrations by heating might be linked with the degradation of immunogenic epitopes, it may be wise to keep the maximal concentration at 20 ppb and to expand the exposure duration, possibly to 1 h. For the initial concentration/dose the present study suggests that lower doses should be used in order to prevent severe reactions in highly susceptible individuals.

Most authors used multiple-steps-challenge protocols that take several days (Malo et al. 1999; Kopferschmitt-Kubler et al. 1998; Vandenplas et al. 1992a; Banks et al. 1989). The test duration in these studies amounts up to 4 days, while the time of exposure varied from 20 to 240 min. Whether 1-day protocols are of sufficient safety needs further study. The comparable lung function impairment of early and late reactions in this study argues for 2-days protocols. Although all reactions were well controlled by bronchodilators, the severity of reactions in this study was considered not acceptable.

It is one of the most important findings of this study that bronchial reactions did not occur later than 2 h after the end of the challenge. Pauses of 90 min between steps were chosen in the present study after 10 and 20 ppb steps. In view of the higher risk of low concentration steps, a pause should be included after the 5 ppb step as well, the duration of the pauses was probably adequate. With pauses of 60–90 min 1-day protocols may be designed with both low initial doses and maintaining dose augmentation by doubling doses. Such protocols have the advantage of lower costs and also a lower chance to produce difficult-to-explain non-linear dose response

relationships of multiple-days protocols (Malo et al. 1999).

The overall low sensitivity of challenge testing in this study of 15 % is probably mainly due to selection, but also false negatives may have occurred, as described earlier (Vandenplas et al. 1992b). That is especially true for doubtful test results in a considerable number of subjects in this study (n = 19; 20 %). As FEV_1, but not specific airway resistance which is less dependent on cooperation of the patient, varied widely in this group, it is obvious that most subjects were in fact negatives. However, as some uncertainty remains, this group was excluded from further analysis. We preferred to include body plethysmography as an effect parameter because spirometry was considered less reliable in this group of subjects who were examined for compensation purposes. Overall, a combination of spirometry and body plethysmography as effect parameters was considered useful, but probably the inclusion of further effect parameters, like serial methacholine testing or the measurement of exhaled nitric monoxide may increase the sensitivity of diisocyanate challenges.

There was no preponderance of a specific profession among responders, but there was an association between the previous degree of exposure to diisocyanates as assessed by expert rating with the responder status. This is highly plausible and points to an overall reliable test system. There was also an association between responder status and bronchial hyperresponsiveness: 86 % of the responders demonstrated bronchial hyperresponsiveness whereas this percentage was much lower in doubtful or negative reactors (32 and 53 %). This was also reflected in a higher number of subjects on current asthma medication in the responders. The most probable explanation is selection of the subjects, most of them had only negligible exposures to diisocyanates in the past, and work-related symptoms were not suggestive of OA due to diisocyanates.

Overall, this study indicates that lower initial doses should be used and that the need for follow-up monitoring longer than 2 h after negative challenges may be abandoned. Whether the compromise between time and effort, on the one hand, and safety, on the other hand, may be obtained with 1-day protocols needs further evaluation.

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

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Occupational Allergic Respiratory Diseases in Garbage Workers: Relevance of Molds and Actinomycetes

42

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Abstract

Exposures to molds and bacteria (especially actinomycetes) at workplaces are common in garbage workers, but allergic respiratory diseases due to these microorganisms have been described rarely. The aim of our study was a detailed analysis of mold or bacteria-associated occupational respiratory diseases in garbage workers. From 2002 to 2011 four cases of occupational respiratory diseases related to garbage handling were identified in our institute (IPA). Hypersensitivity pneumonitis (HP) was diagnosed in three subjects (cases 1–3, one smoker, two non-smokers), occupational asthma (OA) was diagnosed in one subject (case 4, smoker), but could not be excluded completely in case 2. Cases 1 and 2 worked in composting sites, while cases 3 and 4 worked in packaging recycling plants. Exposure periods were 2–4 years. Molds and actinomycetes were identified as allergens in all cases. Specific IgE antibodies to *Aspergillus fumigatus* were detected exclusively in case 4. Diagnoses of HP were essentially based on symptoms and the detection of specific IgG serum antibodies to molds and actinomycetes. OA was confirmed by bronchial provocation test with *Aspergillus fumigatus* in case 4. In conclusion, occupational HP and OA due to molds occur rarely in garbage workers. Technical prevention measures are insufficient and the diagnosis of HP is

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_42,

often inconclusive. Therefore, it is recommended to implement the full repertoire of diagnostic tools including bronchoalveolar lavage and high resolution computed tomography in the baseline examination.

Keywords
Asthma • Garbage handling • Hypersensitivity pneumonitis • Molds • Occupational diseases

1 Introduction

Garbage is of high economic and ecologic worth. In Germany, garbage is collected separately and sorted (e.g. metals, sales packaging, glass, paper, electronic devices, and chemicals) for recycling. The 'green dot' DSD-company handles predominantly used sales packaging, e.g. from households. Despite high standards of environmental protection as well as protective measures for the workforce, the risk of exposure to bioaerosols remains. Neumann et al. (2002) detected 10^2–10^4 colony forming units (CFU) of molds per 1 m^3 air at workplaces of garbage workers and up to 10^7 CFU/m^3 during filling processes of garbage. In compost facilities, up to 10^5 CFU of molds per m^3 were detected (Peersons et al. 2010).

Garbage workers may suffer from asthma due to various causes (e.g., bioaerosols, dust, or volatile organic compounds), but the literature shows no evidence of increased risk of allergic asthma in compost workers (Bünger et al. 2000; Schlosser et al. 2009; van Kampen et al. 2012) and only moderate evidence of increased risk of occupational asthma (OA) in garbage workers (Kuijer et al. 2010). Also, only a few cases of hypersensitivity pneumonitis (HP) have been described in compost workers (Bünger et al. 2007; Weber et al. 1993; Vincken and Roels 1984; Schlosser et al. 2009). The aim of this study was to appraise cases of garbage workers with respiratory diseases who were examined due to suspected occupational disease in the outpatient clinic of our institute (IPA) since 2002.

2 Methods

The study was performed in conformity with the Declaration of Helsinki of the World Medical Association and the protocol was approved by a local Ethics Committee.

Medical examinations were reconstructed based on the files of the accident insurance. The subjects were asked by an occupational physician for work-related symptoms, exposures to bioaerosols at their workplaces, atopic diseases, and smoking habits. They underwent a physical examination, extensive lung function testing (spirometry, body plethysmography and methacholine testing), and analysis of routine blood parameters. In cases 2–4, total IgE, and specific IgE and specific IgG antibodies to various molds and environmental allergens were measured by ImmunoCAP (Phadia, Freiburg, Germany). According to the manufacturer's recommendations a specific IgE concentration of $\geq$0.35 kU/L ($\geq$CAP-class 1) was considered positive. Specific IgG antibodies were measured with ImmunoCAP (Phadia); elevated IgG concentrations were assumed if the manufacturer's cut-offs were exceeded. Skin prick tests (SPT) were performed with extracts of environmental allergens and molds and actinomycetes from various manufacturers (Bencard, Munich; HAL, Duesseldorf, Germany). Radiological examination included chest X-rays or a computed tomography (CT) of the thorax. Specific inhalation challenges (details see case descriptions) and measurements of exhaled nitric oxide (eNO; NIOX Flex; serial

measurements: NIOX MINO; Aerocine, Solna, Sweden) were performed in accord with ATS/ERS recommendations.

3 Results

3.1 Compost Plants

3.1.1 Case 1

A 53-year-old male Caucasian smoker (20 cigarettes per day) worked in a composting plant from 1991 to 1996. He filled garbage chambers and evacuated them after a 7 days decomposing process. He used respiratory protection irregularly. Since 1993, the man experienced dyspnea on exertion, sometimes cough and phlegm. Physical examination demonstrated inspiratory crackles. X-rays of the thorax showed 'spotted interstitial markings' in both lungs. A diagnosis was not provided at that time. Two years later the symptoms persisted. Vital capacity of 65 % predicted was found, blood gas content was normal. IgG antibodies to *Saccharopolyspora rectivirgula* (38 kU/L; manufacturer's cut-off 25 kU/L), *Thermoactinomyces vulgaris* (59 kU/L; manufacturer's cut-off 25 U/mL) and *Candida* (74 kU/L; manufacturer's cut-off 25 kU/L) were positive, whereas no IgG antibodies to *Aspergillus fumigatus* and *Mucor spp*. were found. Repeated X-rays of the thorax revealed a progression of the interstitial markings. A diagnosis of HP was made and the patient was advised to quit his job.

In 1997, 1 year after quitting the job, the patient still suffered from dyspnea on exertion, but had no cough. Physical examination showed weak inspiratory crackles, but spirometry, body plethysmography and CO diffusion capacitance as well as chest X-rays were in normal range. Methacholine testing was negative. SPT with common environmental allergens and intradermal tests with molds indicated a weak reaction with *Fusarium culmorum*, but specific IgE or enhanced specific IgG antibodies concentration to *Saccharopolyspora rectivirgula*, *Thermoactinomyces vulgaris*, *Thermopolyspora polyspora*, and *Aspergillus fumigatus* were not detectable. In a workplace-simulating challenge with biocompost in the laboratory lasting 30 min the patient showed no reaction. CT or bronchoscopy were not performed. A final diagnosis of HP was corroborated.

3.1.2 Case 2

A 41-year-old Pakistani was a never-smoker. Until his immigration to Germany in 1992 he worked in an office. In 1997 he started to work as a biological waste sorter in a composting plant; occasionally he worked as a cleaner in the same factory. Paper masks were used regularly. Since 1994 the patient suffered from upper airways infections. Fever and fatigue were manifest after working hours in 2000, whereas during weekends and holidays the complaints diminished. Since 2001 he suffered from chronic sinusitis. X-rays showed swelling of the mucous membranes of sinuses, but the thorax was normal. SPT with environmental allergens and molds gave no reactions. Spirometry revealed a reduction of FEV_1/VC of 69 %. However, this test appeared qualitatively insufficient and ignored the ATS criteria. Dyspnea on exertion, flu-like symptoms, fever and shivering developed in 2003. Antiobstructive medication was used on demand since then. In 2005 he quit his job and became unemployed.

In 2006 the patient was examined at the IPA. He was symptom-free and without medication. Physical examination, laboratory investigations, and CT were normal. X-rays of the nasal sinuses showed a swelling of mucous membranes. Spirometry was normal, but methacholine testing revealed bronchial hyperresponsiveness. CO diffusion capacity and blood gases were normal. Skin prick tests were positive with *Aspergillus fumigatus*, *Penicillium expansum* and *Penicillium chrysogenum*, but not with environmental allergens from various manufacturers. Elevated specific IgE antibody to *Aspergillus fumigatus* (62 mg_A/L; cut-off 39 concentrations to *Aspergillus fumigatus* (11 kU/L) and enhanced specific IgG antibody concentrations mg_A/L) and *Penicillium chrysogenum* (47 mg_A/L; cut-off 27 mg_A/L) were detected. Total IgE was 637 kU/L. A final diagnosis of HP was made, but a concomitant OA could not be excluded.

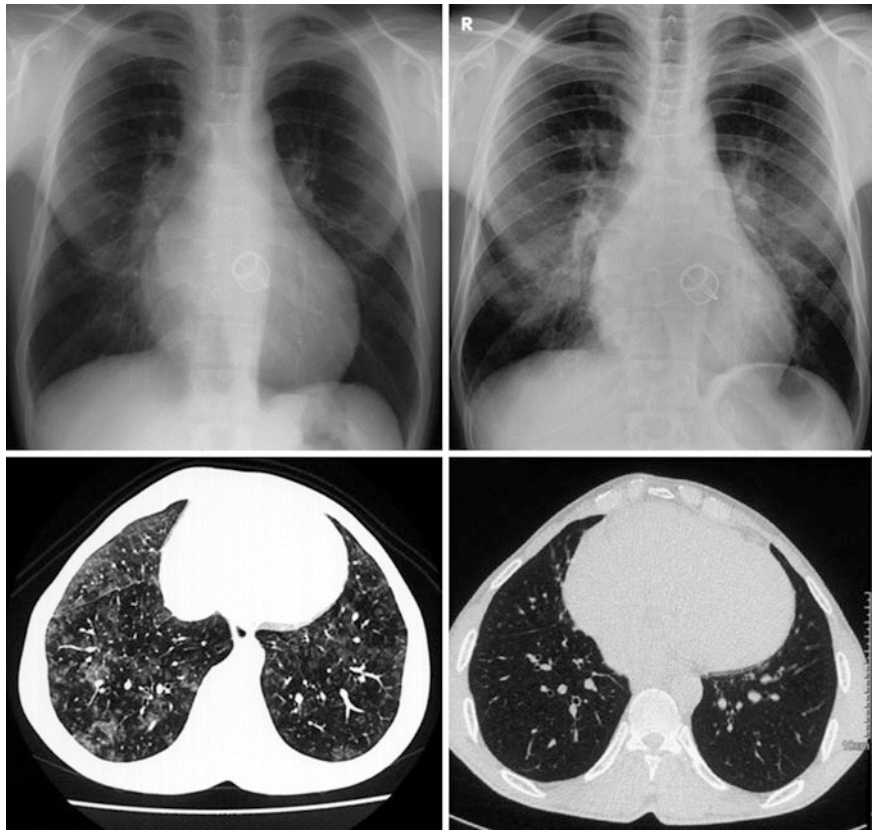

Fig. 42.1 X-rays of the patient (Case 3) in July 2001 (*top left*), January, 2005 (*top right*), November 2004 (*bottom left*), and January 2006 (*bottom right*). In July 2001, a normal picture of lungs with a synthetic mitral valve can be seen. Four years later, the lungs show diffuse ground-glass opacities, and in the CT patchy infiltrates, ill-defined centrilobular nodules, and air trapping. One year after exposure cessation in January 2006, the CT of the lungs is normal again

3.2 DSD-Plants

3.2.1 Case 3

A 33-year-old, never smoking Tamil worked as a garbage sorter of packaging material in a DSD company from 1998 to 2004. Respiratory protection (FFP2 masks) was changed every day. In 1989, the patient got a replacement of his mitral valve. Since 2001 he developed fever, joint pain, cough with dark expectoration, and dyspnea on exertion. Symptoms occurred occasionally in the evenings after work days and diminished during vacations.

In 2004, symptoms increased and the patient lost body weight (10 kg in 3 months). Medical examination showed an absolute arrhythmia, but no rales in the lungs on auscultation. X-rays of the thorax presented diffuse ground-glass opacities in both lungs and CT showed patchy infiltrates, ill-defined centrilobular nodules and air trapping (Fig. 42.1). An increased number of lymphocytes was found in bronchoalveolar lavage (BAL), but no quantitative analysis was performed. A diagnosis of HP was made and the patient was advised to quit his job.

In 2006, about 1 year after exposure cessation, the patient complained of mild dyspnea on exertion, but was otherwise symptom-free. Physical examination of the lung was normal, arrhythmia absoluta was corroborated by electrocardiography. SPT with common environmental allergens and molds were negative, as were specific IgE

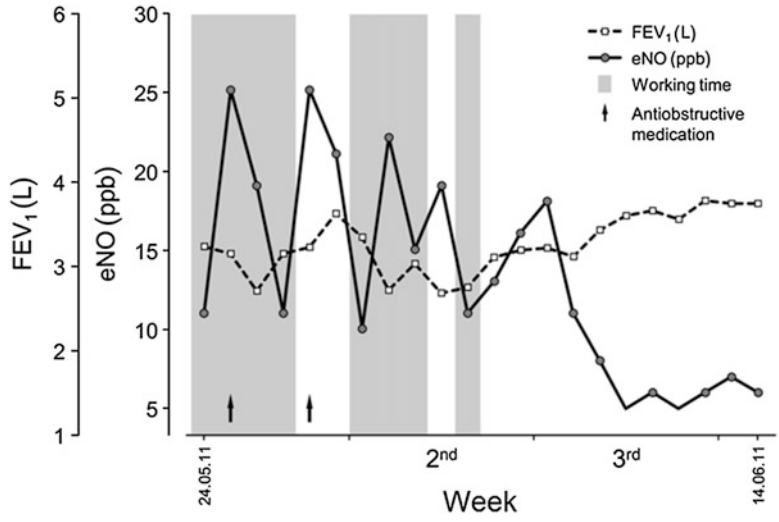

Fig. 42.2 Serial measurement of FEV_1 and eNO. *Grey areas*: working days, *white areas*: leisure time. After starting work, eNO increases and FEV_1 decreases, whereas in leisure time eNO decreases and FEV1 increases

antibodies. IgG antibodies concentrations to *Aspergillus fumigatus* (39 mg_A/L, manufacturers cut off: 39 mg_A/L) were normal and minimally elevated to *Penicillium chrysogenum* (41 mg_A/L, manufacturers cut off: 27 mg_A/L). Spirometry and methacholine testing were normal, but CO diffusion capacity was slightly reduced (70 % predicted). Exercise testing showed normal blood gases. X-rays and CT of the lungs were normal (Fig. 42.1). A final diagnosis of HP was made.

3.2.2 Case 4

A 36-year old Caucasian smoker (10–20 cigarettes per day) worked as a driver in a DSD garbage plant from 1996 to 2002. In 2002 he started his work as a garbage sorter of packaging material. Since 2010 he worked again as a driver. Respiratory protection was never used. The patient was healthy without any allergic diseases until 2000 when he noticed rhinitis, later cough and dyspnea during work and post-shift. There was no history of fever. His otolaryngologist recorded multiple sensitizations to common environmental allergens by SPT and specific IgE concentrations to *Alternaria tenuis* (0.6 kU/L), rye grass (1.8 kU/L) and timothy grass (1.8 kU/L). Symptoms aggravated during the following years with improvement during vacations.

In 2010, the patient was referred to a pneumologist. Airway resistance was 0.34 kPa*s/L (<0.30). Specific IgG to *Trichophyton* spp. was slightly elevated (56 mg_A/L; cut-off >30 mg_A/L). FEV_1 was 70 % predicted and FEV_1/VC was 68 %. CO diffusion capacity was normal. CT of the thorax was normal. A diagnosis of asthma was made and antiobstructive medication was prescribed. Another examination performed by an otolaryngologist showed elevated specific IgE concentrations to *Aspergillus fumigatus* (26.9 kU/L), *Alternaria alternata* (5.2 kU/L), *Dermatophagoides pteronyssinus* (1.1 kU/L), *Dermatophagoides farinae* (1 kU/L), and timothy grass pollen (3.1 kU/L).

The patient was examined in May of 2011 without prior exposure cessation. He suffered from dry cough and variable work-related shortness of breath. Physical examination was normal. SPT were positive with *Alternaria alternata* (5 mm wheal diameter), *Aspergillus fumigatus* (4 mm), and small wheal diameters were recorded with house dust mites, grass pollen and ribwort. Total IgE was 166 kU/L. Specific IgE antibodies to mold mixture (5 kU/L), *Aspergillus fumigatus* (7 kU/L), *Alternaria alternata* (2 kU/L), and to various mites and grass pollen were detected. X-rays of the thorax were normal. Spirometry showed a mild obstructive pattern (FEV_1 78 % predicted). Methacholine testing demonstrated bronchial hyperresponsiveness with a $PD_{20}FEV_1$ of 76 µg methacholine. Monitoring of daily FEV_1 and eNO showed a decrease of eNO and an increase of FEV_1 after cessation of exposure

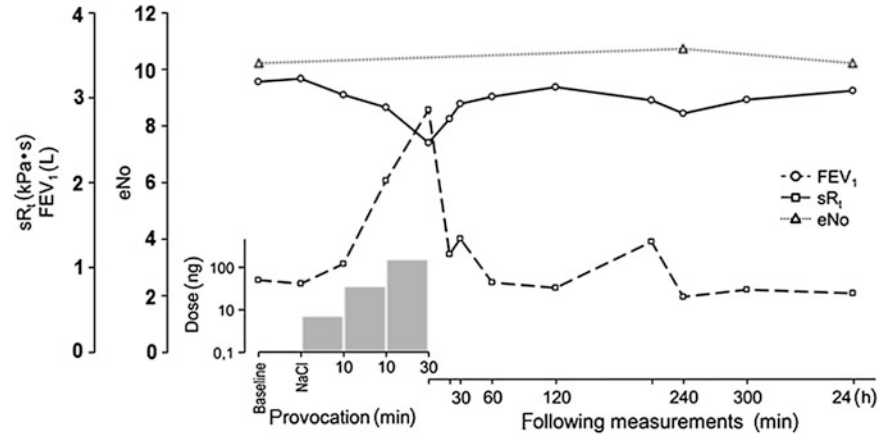

Fig. 42.3 Specific inhalation test with Aspergillus fumigatus. After cumulative exposure of 15 ng, FEV1 decreased >20 % and sRt increased >2.0 kPa*s. eNO showed no change in 24 h

on June 3, 2011 (Fig. 42.2). The patient reported a decrease of his symptoms, but mild cough and expectoration persisted until reassessment on July 6, 2011. On this day, a specific inhalation challenge was performed.

The inhalative challenge was performed with an *Aspergillus fumigatus* extract from Allergopharma (Reinbek, Germany) and was carried out with a DeVilbiss 646 nebulizer and an APSpro dosimeter (Jäger, Höchberg, Germany) in quadrupling concentrations from 8 to 128 ng protein/mL (cumulative dose of 15 ng protein). Briefly, each dose was administered in ten consecutive slow inspirations from functional residual to near total lung capacity, while the nebulizer was actuated over 0.6 s. Inspiratory airflow was maintained close to 1 $L^{*}s^{-1}$ by observation of a visual scale. The time interval between consecutive steps was 15 min. The nebulizer was actuated 0.5 s after the start of inspiration to ensure a significant airflow upon nebulization. The response was evaluated by spirometry and body plethysmography (Masterlab, Jäger, Höchberg). Follow-up lung function measurements were carried out after 10, 20, 30 min, and then hourly from 1 to 5 h and after 24 h. The patient showed a significant increase of airway resistance from 0.35 to 0.83 $kPa^{*}s^{*}L^{-1}$ and a decrease of FEV_1 from 3,170 to 2,450 mL (22 % from baseline; Fig. 42.3). This was accompanied by shortness of breath. Symptoms and lung function gradually returned to baseline without medication.

After complete exposure cessation in June of 2011, the patient had a follow-up examination in 2012. He suffered from dyspnea on exertion and cough, but reported an important improvement of his symptoms. He inhaled topical steroids daily and short-acting beta agonists about twice per week. Physical examination showed wheezing after forced expiration. SPT reactions remained unchanged. Whereas sensitizations to environmental allergens and *Alternaria alternata* (1.94 kU/L) were unchanged, the concentration of specific IgE to *Aspergillus fumigatus* dropped from 6.5 to 1.8 kU/L. Spirometry showed a mild obstructive pattern (FEV_1 75 % predicted). Bronchial hyperresponsiveness had improved slightly ($PD_{20}FEV_1$ of 147 μg methacholine). A final diagnosis of OA was made.

4 Discussion

Although garbage workers are exposed to high concentrations of airborne microbial organisms, epidemiologic data about health effects on the airways or lungs remain rare. This is also the case for allergic respiratory diseases. Very few cases with HP have been reported in garbage workers in the literature and to our knowledge no cases of allergic OA due to molds. Recently, it has been

reported from Finland that molds from moisture damaged buildings are frequent causes of OA in contrast to Canada where such cases have not been found (Piipari and Keskinen 2005). These discrepancies suggest that diagnostic differences between both countries may exist.

Some diagnostic difficulties can be demonstrated in the four cases described in the present article. Prior diagnoses of OA (Cases 2 and 4) or HP (Cases 1 and 3) were made by the patients' pneumologists. Three out of the four cases (Cases 1–3) had already quit their jobs since at least a year and were more or less symptom-free without lung function impairment. Thus, a complete diagnostic work-up including collection and analysis of BAL fluid or challenge tests was not considered due to ethical reasons. The final diagnoses of HP (Cases 1–3) were based on previous HP typical symptoms, the demonstration of enhanced mold- or actinomycetes-specific IgG antibody concentrations and improvement of symptoms after exposure cessation. In Case 3 we had additional information from CT and BAL. A diagnosis of OA in Case 2 could be excluded as the patient presented signs of both OA and HP. He also presented both specific IgE and IgG antibodies against *Aspergillus fumigatus*. The development of specific IgE and IgG antibodies to the same antigen is unusual. Rydjord et al. (2007) described an association between high mold exposure and the development of IgG antibodies to molds, whereas IgE antibodies were seen more often in low exposure scenarios and in susceptible subjects. IgG and IgE antibodies in that study were found to be correlated inversely. We found only a few case descriptions of combined asthma and hypersensitivity pneumonitis due to mold exposure in the literature. O'Brien et al. (1978) described a case with IgE and IgG antibodies to *Merulis lacrymalis* and diagnosed both asthma and HP. A similar combined diagnosis of asthma and HP due to *Bjerkandarea adusta* was described by Katayama et al. (2008). Only 4 case had not quit his job at the time of our examination and reported recent work related symptoms. Thus we considered performing the challenge testing in this case.

Although the diagnoses of HP in the presented cases may be questioned if stringent diagnostic criteria are applied, we consider the likelihood of a false positive diagnosis as low, as HP in garbage workers have been described (in less detail) also by others (Weber et al. 1993; Vincken and Roels 1984; Schlosser et al. 2009). Work related symptoms and a favorable course after exposure cessation, together with specific IgG antibodies, point to occupational reasons. HP has an overall good prognosis if symptomatic exposure duration is minimized. This is often the case in occupational HP because workers relate symptoms to work and quit their jobs. The primary examination of subjects with HP is often inconclusive and the diagnostic work-up for compensation has to find a compromise between the need for a valid diagnosis and the risk of invasive diagnostic tools in nearly asymptomatic subjects. Thus it is recommended that physicians involved in the primary diagnostic procedure should use the full spectrum of diagnostic tools (Sennekamp et al. 2007), including computed tomography of the thorax and BAL, which demonstrate high diagnostic specificity.

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

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Th1, Th2, Th17, and Regulatory Cytokines in Children with Different Clinical Forms of Allergy

43

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Abstract

In addition to Th2 cells, other T cell subsets, like Th1, Th17, and regulatory T cells are also involved in the pathogenesis of allergic rhinitis, food allergy, and other allergic diseases. The aim of the study was to evaluate a wide profile of cytokines in the plasma from children with pollen/dust and cow milk allergy with the use of a multiplex Cytometric Bead Array (CBA) test. Twenty children with allergy: 11 with pollen/dust and 9 with cow milk allergy were enrolled into the study. For the analysis of INFγ, IL-2, IL-4, IL-6, IL-10, IL-17A, and TNFα levels, a flow cytometric test was used. TGFβ concentration was measured using an ELISA test. Concentrations of almost all of the detected cytokines in the patient's plasma were below 1 pg/ml, with no significant difference between the groups examined. The level of IL-17A was higher in pollen/dust allergy group (median 12.73 pg/ml; 25th percentile-Q1/75th percentile-Q3; 9.28/15.83) in comparison to cow milk allergy group (median 7.86 pg/ml – Q1/Q3; 7.86/14.37). Children with cow milk allergy had a slightly higher concentration of TGFβ than children with pollen/dust allergy (median 28.7 ng/ml – Q1/Q3; 19.77/33.00 vs. 17.84 ng/ml – Q1/Q3; 11.21/23.75, respectively); the difference did not reach statistical significance. We conclude that plasma concentration of Th1, Th2, and Th17 regulatory cytokines are relatively low in allergic children. The CBA test, which could be useful in pediatric practice as it requires a relatively small plasma sample volume, is not

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_43,

sensitive enough to measure low cytokine levels, since its detection limit and the standard curve are not appropriate for the concentrations of these mediators expected in clinical samples.

Keywords
Allergic rhinitis • Cytokines • Flow cytometry • Interferon • Milk allergy • Pollen • T cells • Tumor necrosis factor alpha

1 Introduction

Over the last few decades the prevalence of allergic diseases has dramatically increased. Numerous epidemiological studies have revealed a remarkable increase in the prevalence of all types of allergy, not only pollen/dust allergy, but also food allergy, like cow milk allergy (CMA) (Samolinski et al. 2009; Sicherer and Sampson 2006).

Allergic diseases represent a complex, both innate and adaptive immune response to natural environmental allergens, with Th2 cells and allergen specific IgE predominance, and are characterized by an inflammatory reaction associated with an increased production of Th2 cytokines. These cytokines promote and magnify allergic inflammation. Apart from Th2 cells, other T cell subsets, like Th1, Th17, and regulatory T cells (Tregs), are also involved in the pathogenesis of allergic diseases. According to Ciprandi et al. (2009a), serum interleukin 17A (IL-17A) levels correlate with the allergic inflammation severity. Recent studies highlighted the role of T regulatory cells as suppressors of the Th2 response. The most important regulatory and suppressive cytokines are interleukin-10 (IL-10) and transforming growth factor β (TGFβ). There is strong evidence that both cytokines are involved in the regulation of Th1 and Th2 responses in allergic patients (Akdis et al. 2004; Jutel et al. 2003; Cottrez et al. 2000).

According to our knowledge, the majority of studies focus on the role of few cytokines in the course of pollen/dust allergy. The reports dealing with a large panel of cytokines in the blood from young children with cow milk allergy are very scarce. Nevertheless, it is already known that an interaction between Th2 and Tregs is essential in the development of tolerance to cow milk proteins (Savilahti et al. 2010; Saurer and Mueller 2009).

The aim of this study was to evaluate a wide profile of cytokines in the plasma from children with different clinical forms of allergy. Another goal was to assess the usefulness of a multiplex CBA flow cytometric test for the detection of cytokines in plasma samples from young children.

2 Methods

The study was approved by the Ethics Committee of the Medical University of Warsaw, Poland. Twenty children with allergy were enrolled into the study: 11 children with pollen/dust allergy examined outside of pollen season (mean age 8 ± 3 SD years, 3 girls and 8 boys) and 9 children with cow milk allergy, who were enrolled at the time of diagnosis before the implementation of a diary-free diet (mean age 1 ± 1 year, 5 girls and 4 boys). All children were diagnosed and treated in the Department of Pediatric Pneumology and Allergology, Medical University of Warsaw, Poland. Table 43.1 shows the characteristics of the children with allergy.

Approximately 0.8–1.0 ml of blood collected on EDTA from antecubital vein, which remained after routine diagnostic tests performed in children with allergy, were used for the study. Blood was centrifuged and plasma was frozen in −80 °C until cytokines analysis. The following cytokines produced by different T cell subsets were detected:

Table 43.1 Demographic, clinical, and laboratory characteristics of children with allergy

	Pollen/dust allergy	Cow milk allergy
No. of patients (boys/girls)	11 (8/3)	9 (5/4)
Age (year)	8 ± 3	1 ± 1
Allergy (pollen/dust)	10/5	Not applicable
WBC	8,654 ± 2,411 cells/μl	9,797 ± 2,207 cells/μl
Lymphocytes	3,372 ± 1,107 cells/μl	6,866 ± 2,094 cells/μl
Eosinophils	456 ± 452 cells/μl; 5.2 ± 4.3 %	383 ± 316 cells/μl; 4.0 ± 3.1 %

Values are means ± SD

interferon γ (INFγ), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), IL-10, TGFβ, IL-17A, and tumor necrosis factor (TNFα). All but one cytokine were detected by Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit (Becton-Dickinson, San Jose, USA). The TGFβ concentration was evaluated using an ELISA test.

2.1 Flow Cytometric Cytokines Detection

The CBA test was used to quantitatively determine the cytokine concentration in plasma samples. The preparation of beads, standards, reagents, plasma samples, and the protocols for flow cytometer setup and data acquisition were performed according to the manufacturer's instruction. Briefly, seven beads populations with distinct fluorescence intensity (FL4) were coated with PE-conjugated antibodies specific for the cytokines mentioned above. Next, 50 μl of mixed capture beads coated by a specific antibody were placed into the assay tubes and mixed together with 50 μl of recombinant standards or the patients' plasma. Subsequently, 50 μl of PE Detection Reagent were added to each tube. Then, the tubes were incubated for 3 h at room temperature in darkness. After the incubation, the samples were washed with 1 ml of a buffer and centrifuged for 5 min at 200 × *g*. After adding 300 μl of a wash buffer, the tubes were evaluated in a flow cytometer (Cytomics FC500, Beckmann Coulter, Brea, CA). The results were calculated using an FCAP Array v2.0 software (SoftFlow, Pecs, Hungary) and were expressed in pg/ml.

To calculate the concentrations of cytokines, a ten point standard curve (0, 20, 40, 80, 156, 312.5, 625, 1,250, 2,500, and 5,000 pg/ml) was performed according to the manufacturer's instructions. Figure 43.1 illustrates an example of a four point standard curve and of the calculation performed for a selected cytokine.

2.2 ELISA Test

Analysis of the human TGFβ analysis was performed using a quantitative sandwich enzyme immunoassay technique according to the manufacturer's instructions (DRG Diagnostics, Marburg, Germany). The results were expressed in ng/ml.

2.3 Statistical Analysis

Data are given as median, 25th percentile (Q1) and 75th percentile (Q3). All parameters measured had a nonparametric distribution according to Shapiro-Wilk's criteria. Thus, statistical analysis was performed using a nonparametric Mann Whitney *U* test for independent samples. To assess the correlations between results Spearman's test was used.

3 Results

3.1 Assessment of Cytokines in Plasma by Flow Cytometry

Detection and concentration of all seven cytokines tested by flow cytometry in the plasma

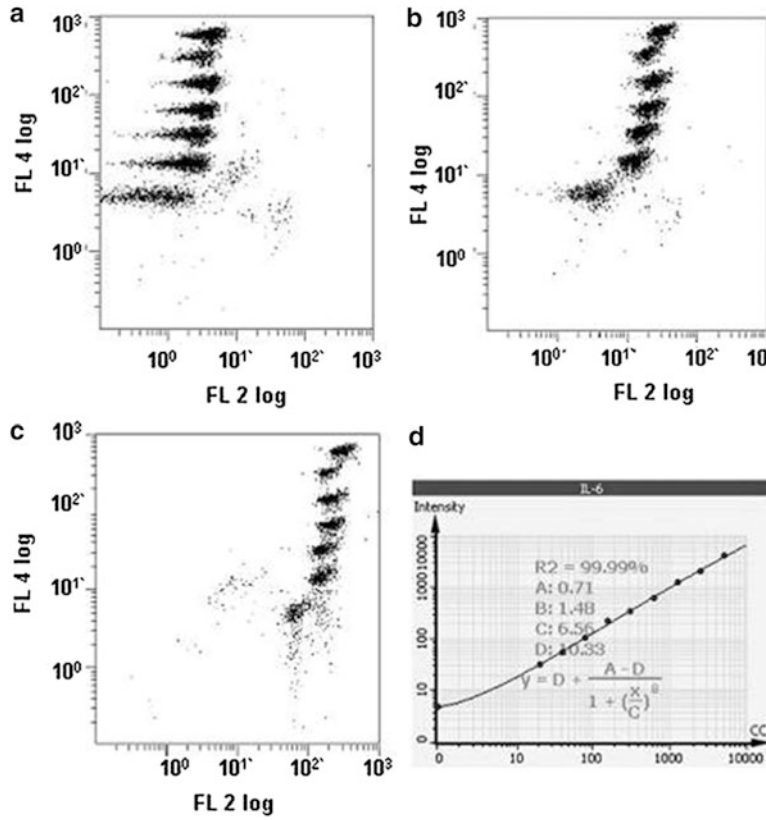

Fig. 43.1 **Examples of three points of a standard curve** (**a**: 20 pg/ml, **b**: 156 pg/ml, and **c**: 2,500 pg/ml). X axis (FL2 log), grouped beads for cytokines; from *top* IL-2, IL-4, IL-6, IL-10, TNFα, INFγ, IL-17A. Y axis (FL4 log), mean fluorescence intensity. *Panel d*, calculated standard curve for IL-6 (x axis, *cc* standard concentration, *y* intensity = mean fluorescence intensity for IL-6 beads)

Table 43.2 Cytokines in plasma of children with allergy

	Children with pollen allergy		Children with cow milk allergy	
Cytokine	Cytokine concentration	No. of samples with a cytokine detected/total	Cytokine concentration	No. of samples with a cytokine detected/total
IL-2	0.44 (0.00/0.93)	8/11	0.31 (0.00/0.53)	5/9
IL-4	0.70 (0.55/1.28)	10/11	0.21 (0.00/1.22)	5/9
IL-6	0.78 (0.42/1.17)	11/11	0.71 (0.36/1.00)	7/9
IL-10	0.77 (0.34/0.95)	10/11	0.70 (0.27/1.21)	7/9
TNFα	1.19 (0.49/1.50)	10/11	0.71 (0.45/1.34)	8/9
INFγ	1.24 (0.00/1.49)	8/11	0.43 (0.00/1.01)	6/9
IL-17A	12.73 (9.28/15.83)	10/11	7.86 (7.86/14.37)	8/9

Values for cytokine concentrations are all means (Q1/Q3) (pg/ml)

of children with pollen and cow milk allergy are shown in Table 43.2.

The cytokine level was undetectable in the majority of the samples examined if adjusted to the lowest 20 pg/ml point of the standard curve. The estimated concentration of the cytokines was below 1 pg/ml. Only were the levels of IL-17A higher in the majority of samples. Statistical differences between groups were not observed. Examples are shown in Fig. 43.2. Dilution of

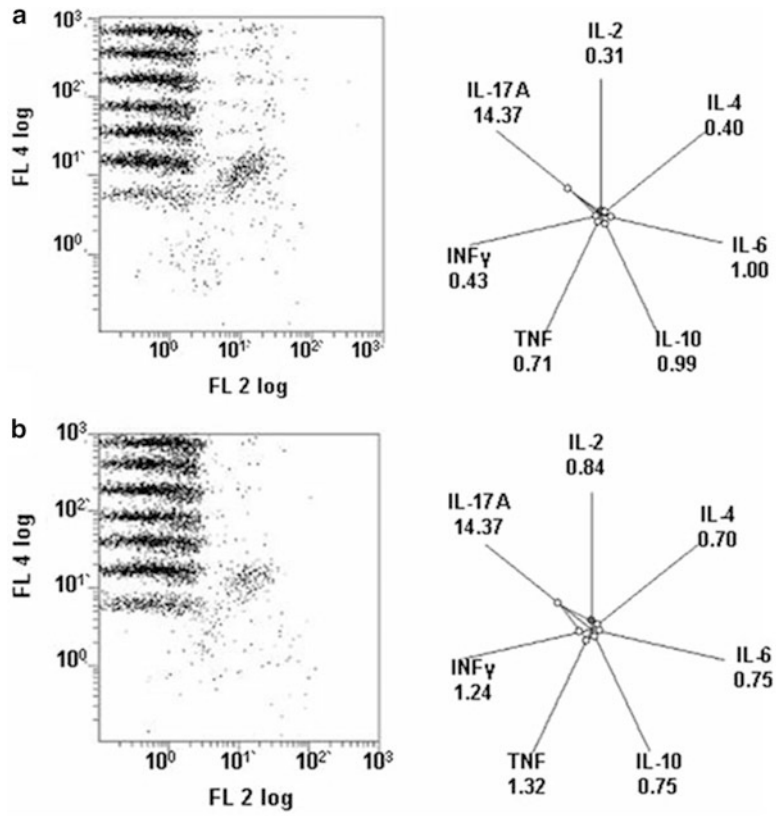

Fig. 43.2 Assessment of seven cytokines in one sample from a patient with pollen (**a**) and a patient with cow milk allergy (**b**) illustrating the use of flow cytometry for the analysis of cytokines (FCAP Array Software). *Dot plot* shows the localization of the cytokines beads (FL4, y axis) and their mean fluorescence intensity (FL2, x axis); radar plots show concentrations of cytokines (pg/ml)

plasma samples before the analysis, according to the manufacturer's instruction, did not influenced the final results (data not shown). There were no correlations between the cytokine concentration in the patients' plasma and white blood cell count, lymphocyte count, lymphocyte percentages, eosinophils count, eosinophils percentages, and sIgE for birch, timothy, or dust allergens.

3.2 Assessment of TGFβ Concentration

The patients with pollen/dust allergy had a mean TGFβ concentration of 17.84 ng/ml (Q1/Q3 – 11.21/23.75) and those with cow milk allergy had TGFβ concentration of 28.7 ng/ml (Q1/Q3 – 19.77/33.00). The children with cow milk allergy had a slightly higher concentration of TGFβ in the plasma than those with pollen/dust allergy (Fig. 43.3). There was no correlation between the level of TGFβ and that of other cytokines tested.

4 Discussion

In the present study, we performed cytokines profiling with the use of the CBA multiplex cytometric assay to quantify a number of cytokines in a small plasma sample and to explore potential differences in children with allergic diseases. This method allows testing six to seven or even more different cytokines in small 50 μl volume of plasma or serum and thus overcomes low tolerance for large volume blood sampling of infants and small children (Tang et al. 2012; Ng et al. 2003). The results reveal that plasma cytokines were below the detection limits in both undiluted and diluted pediatric samples. In contrast, plasma IL-17A levels were readily quantified and were within the dynamic range of the analytical assay system. A major

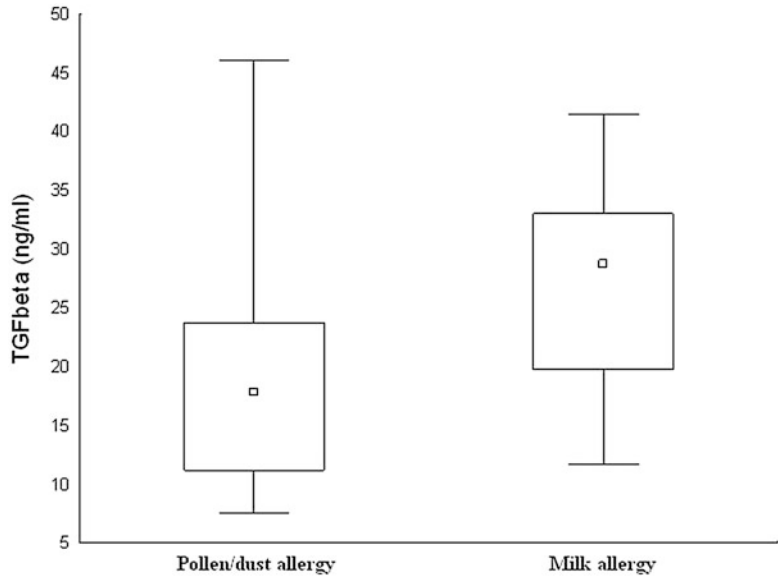

Fig. 43.3 Concentration of TGFβ in peripheral blood of children with pollen/dust (n = 11) and cow milk allergy (n = 9). Results are shown as medians (25th/75th percentiles) and min-max

limitation of the approach used in the present study was related to the standard curve obtained on the basis of high concentrations of standards; the highest concentration was 5,000 pg/ml and the lowest was 20 pg/ml, which might be the reason of decreased sensitivity of the assay. The concentrations of almost all detected cytokines were under 20 pg/ml and needed to be estimated from the 4PL curve. The determination of so low as that concentrations of cytokines by extrapolating the standard curve cannot be sensitive enough, which makes some authors suggest not to give exact values, but instead a statement 'below the lowest standard' (Leng et al. 2008). ELISA assays, on the other hand, may operate within lower concentrations of the analyte, but their usefulness is limited by the need of a large blood volume and by high costs.

Our findings are consistent with those of other authors using more sensitive ELISA tests or CBA tests (Tang et al. 2012; Dabitao et al. 2011). Nevertheless, different authors demonstrated higher concentrations of plasma cytokines, e.g., IL-4 and INFγ in a study of Kirmaz et al. (2011) or IL-6 and IL-17A in that of Wei et al. (2011). Inconsistent results between different studies may be explained by different clinical characteristics of the patients such as age, type of sensitization, or clinical symptoms.

According to the state of the art of the pathogenesis of allergic diseases, one could expect higher concentrations of Th2 cytokines and lower concentration of Th1 cytokines in patients' samples. This was not confirmed in the present study. The discrepancy may be explained by low concentrations of cytokines in the peripheral blood in general. Higher concentration of Th2 cytokines in patients with pollen/dust allergies can be observed in BALF than in plasma (Wei et al. 2011), or in case of cow milk allergy in intestinal tissue (Paajanen et al. 2005). Therefore, it seems suitable to analyze the material derived from the site of a disease, if such samples may be obtainable. In the present study, IL-17A was the only cytokine that was present in a relatively high concentration. This is in agreement with the clinical stage of children with allergy. This observation supports the hypothesis that persistent allergic inflammation is maintained by IL-17A. On the other hand, Ciprandi et al. (2009b) have demonstrated that only two patients out of the 23 included had detectable levels of, measured by ELISA. The IL-17A concentrations were akin to those in the present study; 13.27 pg/ml vs. 12.73 pg/ml, respectively. Wei et al. (2011) have demonstrated high levels of IL-17 in asthmatic patients: 48.3 pg/ml (n = 48). According to Ciprandi et al. (2009a), IL-17A concentration in

plasma can be considered as a new biomarker of disease activity. These authors have demonstrated that IL-17A correlates with the patients' sIgE concentration in plasma and with the peripheral eosinophilic count. However, in the present study we did not observe such a correlation.

IL-10 and TGFβ are known as regulatory cytokines and one may expect a decrease of these mediators. In the present study, IL-10 concentrations were comparable with other analyzed cytokines. Our observations were consistent with the findings of Tsai et al. (2009) and Wei et al. (2011). We also found that TGFβ concentrations corresponded with the findings of other authors (Ciprandi et al. 2009b). Ciprandi et al. (2010) have shown that serum TGFβ levels are dependent on the intensity of allergen exposure. This observation is consistent with our present finding that children with cow milk allergy, before initiating a milk restriction diet, had a slightly higher concentration of TGFβ than children with pollen/dust allergy outside the pollen season. Another possible explanation of this observation may be related to the difference in the age of children in both group examined; cow milk allergic children were much younger than those with pollen allergy.

We conclude that the plasma concentrations of Th1, Th2, Th17, and regulatory cytokines do not differ in children with pollen/dust allergy and with cow milk allergy, and they are relatively low. However, our findings are limited by a relatively small sample size. Further study will be performed to confirm these findings and their clinical relevance. The CBA test seems a useful tool to measure multiple analytes in small pediatric samples. Nevertheless, low concentrations of the mediators examined imply the necessity to generate an appropriate standard to increase analytical sensitivity of the test.

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

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Soluble Receptor for Urokinase Plasminogen Activator in Community-Acquired Pneumonia in Children

44

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Abstract

Community-acquired pneumonia (CAP) is a leading single cause of mortality in children under 5 years of age. In search of new diagnostic markers, soluble urokinase plasminogen activator receptor (suPAR) seems to offer promise as a novel clinical tool. The goal of the present study was to assess the relation between suPAR and the severity of CAP. suPAR was measured in 74 (39 males, 35 females) patients aged from 1 month to about 15 years. Correlation between the level of suPAR and inflammatory markers (white blood cell, neutrophil count, C-reactive protein-CRP, and procalcitonin-PCT) was assessed by Spearmann's rank coefficient. We found that the median suPAR level in children with pneumonia was 8.29 ng/mL (range 2.44–18.31 ng/mL). In the multivariate logit model, age and CRP level were statistically important. The older children (age above the median value) had higher suPAR (above the median value) less frequently than the younger children (OR = 0.31), whereas the children with greater CRP values (above the median value) had higher suPAR levels than the children with lower CRP concentration (under the median value) (OR = 4.54). There was also a positive correlation between suPAR and PCT levels. In conclusion, we demonstrate a positive correlation between serum suPAR and the non-specific inflammatory markers CRP and PCT in the community acquired pneumonia in children.

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_44,

Keywords
Children • Community acquired pneumonia • Inflammatory markers • Soluble urokinase plasminogen activator receptor (suPAR) • White blood cell

1 Introduction

Community-acquired pneumonia (CAP) is a leading single cause of mortality in children under 5 years of age (Rudan et al. 2008) and requires accurate and timely diagnosis. Yet the number and quality of clinical tools is still limited. In contrast to adult patients, there is no widely-accepted and evidence-based scale for rating severity of pneumonia in children. This makes search of new diagnostic and prognostic tools necessary. One of them, soluble urokinase plasminogen activator receptor (suPAR) seems promising. suPAR is a soluble form of the urokinase-type plasminogen activator receptor (uPAR/CD 87) and derives from its proteolytical cleavage and release (Huai et al. 2006). suPAR is present in plasma, cerebrospinal fluid, and urine (De Witte et al. 1998; Garcia-Monco et al. 2002), although most of the studies focused on its serum levels. It is expressed in a variety of immune system cells, like neutrophils, monocytes, and macrophages (Blasi and Carmeliet 2002; Plesner et al. 1997) and seems associated with the immune and inflammatory processes in humans (Ostrowski et al. 2005; Fevang et al. 2009). Enhanced levels of suPAR have been found in bacterial infections, like active pulmonary tuberculosis (with predictive value) (Eugen-Olsen et al. 2002), bacterial meningitis (Ostergaard et al. 2004), or bacteremia (with prognostic value) (Huttunen et al. 2011), and in parasitic (Perch et al. 2004) or viral infections; in HIV infection suPAR is linked with poor prognosis (Sidenius et al. 2000; Lawn et al. 2007). Increased suPAR is also seen in some types of solid tumors (Thuno et al. 2009). uPAR, from which suPAR derives, plays an important role in the innate immune response in the lungs (Wiersinga et al. 2010). Although the value of suPAR as a single marker is limited, it may be of importance when combined with other clinical or laboratory factors (Kofoed et al. 2007). In the present study, we focused on the relation between suPAR and the severity of CAP assessed by routine laboratory parameters. The aim was to investigate the correlation between suPAR levels and CAP severity and the suPAR's potential value in clinical practice.

2 Methods

The protocol of the study was approved by a local Ethics Committee. This prospective analysis was conducted in the Department of Pediatrics of the Medical Center of Postgraduate Education and Bielanski Hospital in Warsaw, Poland.

The subjects of the study were children hospitalized due to pneumonia. To ascertain the presence of community-acquired, and not nosocomial, pneumonia, children with CAP diagnosed within the first 48 h after admission were included. Between February 2011 and March 2012 (13 months), 243 children (8 % of admissions) with pneumonia were hospitalized. Thirty seven children (15.2 %) did not meet the inclusion criteria and were excluded from further analysis. Chest radiographs were taken in 180 out of the 206 (87.4 %) children who met the inclusion criteria, and pneumonia was confirmed in 169 cases (93.9 %). The age of children included in the study ranged from 1 month to about 15 years. The exclusion criteria consisted of respiratory system and musculoskeletal defects or previous medical interventions that could have an influence on CAP course or facilitate infection (e.g., tetraplegia, lung decortication), previously diagnosed proliferative disease, diabetes mellitus, heart, renal, thyroidal, hypophyseal, or adrenal insufficiency.

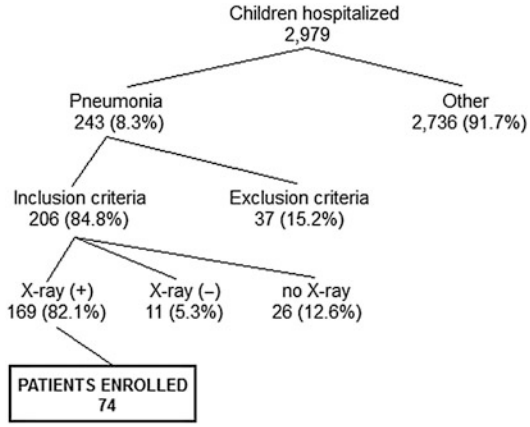

Fig. 44.1 Structure of hospitalizations 2011–2012 and patients enrolled

Serum from every admitted patient was collected on admission and frozen to perform ELISA test later on (suPARnostic, Virogates). suPAR was measured in 74 (39 males, 35 females) out of the 169 (43.8 %) patients (Fig. 44.1). A low percentage of suPAR measurements resulted from a low number of serum samples that were left for the study after routine blood sampling. Not to put an extra burden on the patients, the ethical approval obtained did not give a provision of collecting more blood than needed for routine laboratory tests. Since there is no widely-accepted and evidence-based scale for rating the severity of pneumonia in children, we used serum inflammatory markers (white blood cells count, neutrophil count, serum C-reactive protein, and procalcitonin) as reference.

Statistical analysis was performed using Statistica 10 (by Statsoft). Shapiro-Wilk test was used to determine whether data were distributed normally. To assess the correlation between suPAR levels and inflammatory markers, Spearmann's rank correlation coefficient was used. $P < 0.05$ was considered as statistically significant. The correlation between suPAR and serum inflammatory markers was also assessed using multivariate logit model. suPAR, WBC, neutrophil, and lymphocyte counts, and C-reactive protein (CRP) and procalcitonin (PCT) levels were categorized using median values. Patients' gender and age were also included in this model. The goodness-of-fit for this model was checked with Hosmer-Lemeshow test. The correlation between suPAR and inflammatory markers was measured as odds ratio (OR) of higher suPAR levels (above median value) for patients with higher (above median value) or lower (under median value) levels of inflammatory markers.

3 Results

The median suPAR level in the children with pneumonia was 8.29 ng/mL; range 2.44–18.31 ng/mL (Table 44.1). Rather weak, albeit significant, positive correlations were found between suPAR and CRP and suPAR and PCT ($r = 0.25$; $p < 0.05$). Other correlations (suPAR-WBC and suPAR-neutrophil count) were of no statistical significance (Table 44.2).

In the multivariate logit model, age and the level of C-reactive protein were statistically important. The older children (age above the median value) had higher suPAR (above the median value) less frequently than the younger children (OR = 0.31), whereas the children with greater CRP values (above the median) had higher suPAR levels (above the median value) than the children with lower CRP concentration (under the median value) (OR = 4.54) (Table 44.3).

There was a dissonance consisting of rather low suPAR correlations in two patients: an 8-year old girl had suPAR of 6.718 ng/mL with highly elevated CRP of 257 mg/L, (standard value <5.0) and slightly elevated PCT of 0.62 ng/mL, (standard value <0.50), while just a slightly higher suPAR of 6.98 ng/mL was present in a 6-year-old boy with highly elevated CRP of 168.7 mL/L and also very high PCT of 29.72 ng/mL.

4 Discussion

In the present study, we demonstrate that there was a positive correlation between serum suPAR and the non-specific inflammatory markers CRP

Table 44.1 suPAR and inflammatory markers concentrations in children enrolled into the study

	Median value	Minimum	Maximum	95 % Confidence interval
suPAR (ng/mL)	8.29	2.44	18.31	(2.46; 3.40)
WBC (*10^3/uL)	14.80	3.60	48.40	(7.32; 10.14)
Neu (*10^3/uL)	8.44	0.39	33.88	(6.12; 8.48)
Ly (*10^3/uL)	3.02	0.34	27.90	(3.46; 4.80)
CRP (mg/L)	34.11	0.10	310.17	(75.06; 104.29)
PCT (ng/mL)	0.36	0.01	29.72	(3.68; 5.17)

Table 44.2 Spearmann's rank correlation coefficient for suPAR and serum inflammatory markers

	Correlation coefficient	p
suPAR-WBC	−0.027	NS
suPAR-Neu count	−0.032	NS
suPAR-Ly count	−0.037	NS
suPAR-CRP	0.255	<0.05
suPAR-PCT	0.252	<0.05

NS nonsignificant

Table 44.3 Oddss ratio value for suPAR-age and suPAR-CRP correlations

	Odds ratio	95 % Confidence interval	p
suPAR-age	0.31	(0.1; 0.93)	0.037
suPAR-CRP	4.54	(1.5; 13.8)	0.008

and PCT in the community acquired pneumonia in children The power of these correlations seems rather low to be of clinical importance in the evaluation of the severity of CAP, although in case of suPAR vs. CRP the calculated OR was 4.54.

One of the limitations of this study is that the suPAR levels were correlated just with the serum inflammatory markers. This approach resulted from a traditional use of those markers for the assessment of infection severity. It was thus the aim of the study to use the routine laboratory markers for the investigation of correlations with suPAR. Taking into account that combined information from several markers could more reliable as a diagnostic tool than a single one (Kofoed et al. 2007), the method we used may not reflect a real role of suPAR in CAP. suPAR increases during bacterial, viral, and parasitic infections and reflects the level of activation of the immune system, regardless of etiology (Backes et al. 2012). CRP, and especially PCT, on the other hand, is more likely to increase during bacterial infections (Li et al. 2011); hence, weak correlations between suPAR and CRP or PCT might be caused by differences in infectious background. In this study we did not elaborate on agents causing pneumonia and the patient group consisted of children with just radiologically confirmed CAP.

Because of possible affinity of uPAR to pulmonary processes, suPAR might be of higher value in diagnosing pneumonia than other inflammatory markers. uPAR, from which suPAR derives, facilitates recruitment of leukocytes toward the site of infection in pneumonia caused by *Pseudomonas aeruginosa* or *Streptococcus pneumoniae* (Gyetko et al. 2000; Rijneveld et al. 2002). Moreover, uPAR-knockout mice shows reduced neutrophil migration and impaired phagocytosis in the lungs (Wiersinga et al. 2010). Thus, deficiency of uPAR may have to do with a poorer course of pneumonia. It remains to be evaluated if patients with severe pneumonia have suPAR concentrations lower than expected. In our study, two patients had unexpectedly low suPAR concentrations, compared with the remaining children with CAP, which did not correspond with CRP and PCT levels. If these findings were associated with uPAR/suPAR deficiency, the children would be at a possibly higher risk of pneumonia.

Another limitation of this study was that there was no control group. Although there is a shortage of data on suPAR in children for a meaningful comparison, we may say that the values we

found in the present study were fairly high. In a study by Wittenhagen et al. (2011), healthy children have a median plasma suPAR concentration of 2.3 ng/mL, while the suPAR level in children with urinary tract infection, and scintigraphically confirmed renal involvement, was 7.3 ng/mL. Those authors concluded that a relatively small increase in suPAR during infection may result from its being less fluctuating in comparison with other markers, such as CRP. Then, a twofold increase in suPAR concentration is enough to predict inflammation. This supposition makes an interesting concept that requires to be confirmed in further studies. To this end, a study comparing children with radiological lung infiltrations versus respiratory tract infections without radiologically confirmed pneumonia would be highly contributory.

Acknowledgments Supported by grant 502-1-20-01-12 of the Medical Center of Postgraduate Education in Warsaw.

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

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Hypersensitivity Pneumonitis due to Metalworking Fluids: How to Find the Antigens

45

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Abstract

Most surveys of outbreaks of hypersensitivity pneumonitis (HP) in subjects with occupational exposure to water-based metalworking fluids (MWFs) were unable to detect a clear link between symptoms and the precise causative agents. We studied the case of a male 41-year-old industrial knife grinder with exposure to water-based MWFs since 12 years. The diagnosis of HP was made by typical work-related symptoms, the demonstration of high lymphocyte numbers in bronchoalveolar lavage and elevated IgG antibody concentrations to various molds in the patient's serum, and complete recovery after early exposure cessation. Whereas an environmental survey showed only low numbers of mold contamination in one sump sample, high antigenic activity was demonstrated in the same sample by antigen-specific IgG inhibition tests. We conclude that the detection of antigenic molds in water-based MWFs by culture methods may be limited. The link between occupational exposure to specific molds in MWFs and hypersensitivity pneumonitis can be established by the demonstration of antigenic activity by antigen-specific IgG inhibition tests.

Keywords

Actinomycetes • Bronchoalveolar lavage • Hypersensitivity pneumonitis • Metalworking fluids • Molds • Occupational exposure

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_45,

1 Introduction

Cases with hypersensitivity pneumonitis (HP) and occupational exposure to water-based metal-working fluids (MWFs) were reported repeatedly in literature (Robertson et al. 2007; Fishwick et al. 2005; Hodgson et al. 2001; Fox et al. 1999). However, environmental surveys were unable to detect a clear link between case definitions and qualitative exposure indices in most symptomatic workers. We report such a case by using a novel method in order to avoid pitfalls of hitherto existing diagnostic tools.

2 Case History

A 41-year-old industrial knife grinder with exposure to water-based MWFs since 12 years developed recurrent work-related 'flu-like' symptoms, fatigue, joint pain, and mild shortness of breath. A diagnosis of HP was made by his pneumologist about 2 months later. Whereas lung function and high resolution computed tomography were normal, bronchoalveolar lavage yielded 65 % lymphocytes, and elevated specific IgG antibody concentrations to various molds were found in the patient's serum. After immediate complete exposure cessation the patient was seen in IPA for a medical opinion shortly after the results of the environmental survey became available (see below). He had no symptoms and lung function was normal. The subject gave written informed consent to publish his case.

3 Methods

3.1 Environmental Survey – Sampling

A survey in the working environment was performed by the Statutory Accident Insurance of the Wood and Metal Industry a few weeks after the diagnosis. The patient had worked on grinding machines with separate MWFs sumps. Samples were taken from the sump of a machine without visible contamination (MWF1) and from the sump of a machine with visible snow flurry-like contamination (MWF2; two samples were taken from MWF2 with a 3 weeks interval). This machine was inoperative since about 2 months prior to the survey for technical reasons. Two different water-based MWFs, but no additional biocides were used in this plant.

Water-based MWFs samples were collected in sterile 500 mL bottles for the determination of colony counts and identification of bacteria and molds. MWF samples were transported to the laboratory within 24 h at temperatures of 2–8 °C. Microbiological analyses were carried out immediately after arrival of the samples.

3.2 Environmental Survey – Analyses of Bacteria

For the determination of total colony counts samples were serially diluted with 0.9 % NaCl and inoculated on tryptic-soy agar (TSA; Merck, Darmstadt, Germany) with 0.3 g/L cycloheximide (AppliChem, Darmstadt, Germany). Plates were incubated aerobically at 30 °C. Colonies were counted after 24 h up to 6 days. Predominant colonies on the total-count plates were picked and grown as a pure culture for further identification. Colony characteristics or morphological characteristics of spores were described as recommended by Bergey's Manual of Determinative Bacteriology (Holt 1994). Selected bacterial isolates were Gram-stained and further identified using the Crystal-System GP (Becton-Dickinson Diagnostics, Heidelberg, Germany) and the API-System 20NE (bioMérieux, Nuertingen, Germany). For total colony counts of thermophilic actinomycetes, samples were serially diluted with 0.9 % NaCl and 0.01 % Tween 80 and inoculated on glycerol-arginine agar (Danneberg and Driesel 1999). Plates were incubated at 36 °C and colony counting was performed up to 14 days. Mycobacteria were detected by microscopy and cultivation as described earlier (Medical Standards Committee 1996). Identification of certain species was carried out by polymerase-chain-reaction (PCR) and the detection of specific gene-sequences (Khan and Yadav 2004).

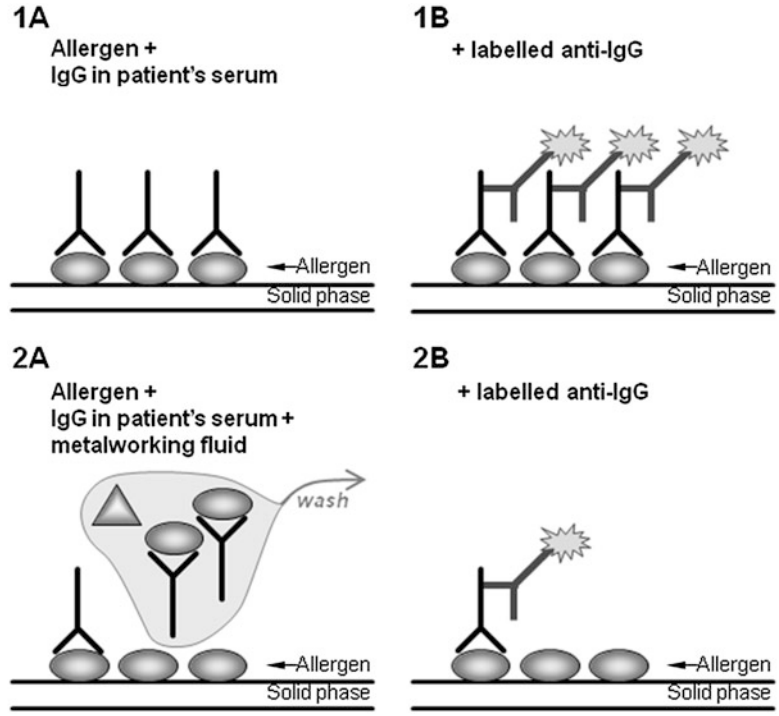

Fig. 45.1 Principle of IgG inhibition testing with metalworking fluid. Diagrams (**1A**) and (**1B**) show the direct binding of IgG antibodies in the patient's serum to the allergens bound to the solid phase. Preincubation with metalworking fluid in inhibition testing (**2A**) yields lower binding of IgG to the solid phase (**2B**). The binding differences (between **1B** and **2B**) are expressed as inhibition rates

3.3 Environmental Survey – Analyses of Molds

For the determination of total colony counts samples were serially diluted with 0.9 % NaCl and 0.01 % Tween 80 and inoculated on dichloranе-glycerol agar (DG 18, Oxoid, Wesel, Germany). Plates were incubated aerobically at 25 °C. Colonies were counted after 48 h, then every 24 h up to 7 days. Mold species were identified by microscopy and visual detection of conidia. For the detection of *Aspergillus fumigatus*, samples were inoculated on malt extract agar (Merck, Darmstadt, Germany) and incubated at 37 °C.

3.4 Immunological Testing

Specific IgG antibodies to various microorganisms were measured with IgG-ImmunoCAP (Phadia, Freiburg, Germany) in the patient's serum. Sump samples from MWF1 and MWF2 were used to inhibit binding of the patient's specific IgG antibodies to commercially available ImmunoCAPs. The method has been described in detail recently (Merget et al. 2009). Briefly, the sump samples were centrifuged at 2,400 × *g*, the pellets were homogenized by Precellys SK38 (Bertin, Montigny-le-Bretonneux, France) at 3 × 6,500 rpm, centrifuged at 15,800 × *g*, and the sterile filtrated supernatant was used for specific IgG inhibition (Fig. 45.1).

4 Results

4.1 Environmental Survey

The first MWF2 sample contained 6.3×10^2 colony forming units (CFU) of molds/mL (predominantly *Fusarium solani* and *Fusarium oxysporum*) and 1.2×10^6 CFU of bacteria/mL (predominantly *Micrococcus luteus* and *Mycobacteria* sp.), the second MWF2 sample showed similar contamination but was not cultivated. MWF1 did not show any growth of molds, but yielded 2.2×10^5 CFU of bacteria/mL (*Moraxella* sp. and *Mycobacteria* sp.). In none of the MWF samples thermophilic actinomycetes were detected. Air monitoring

Table 45.1 IgG antibody concentrations in the patient's serum and inhibition rates of IgG-binding to different microorganisms with sump samples of a grinding machine without (MWF1) and with (MWF2) visible snow flurry-like contaminations

Antigen	IgG-antibody concentration (mg/L)	Manufacturer's cut-off (mg/L)	Inhibition with MWF1 (%)	Inhibition with MWF2 (%)[a]
Alternaria alternata	70	12	0	63
Fusarium proliferatum	133	46	8	48
Aspergillus fumigatus	78	39	0	13
Aspergillus versicolor	38	100	ND	ND
Cladosporium herbarum	154	37	0	55
Penicillium sp.	85	50	ND	ND
Aureobasidium pullulans	123	22	8	78
Saccharopolyspora rectivirgula	53	10	5	27

ND not done, [a]MWF2 inhibition rates represent the arithmetic mean of two separate samples

did not show microbial contamination (data not shown). In summary, the environmental survey yielded very low mold contamination of MWF2.

4.2 Immunological Testing

The earlier findings of elevated specific IgG antibodies to molds in the patient's serum were reproduced. More than twofold higher specific IgG antibody concentrations than the manufacturer's cut-off values were detected with *Alternaria alternata* (Gm6), *Fusarium proliferatum* (m9), *Cladosporium herbarum* (Gm2), *Aureobasidium pullulans* (m12) and the actinomycete *Saccharopolyspora rectivirgula* (Gm22). Specific IgG inhibition experiments showed no inhibition with MWF1, but the preincubation of the patient's serum with MWF2 strongly inhibited the binding of the specific IgG antibodies to antigens of *Alternaria alternata*, *Fusarium proliferatum*, *Cladosporium herbarum* and *Aureobasidium pullulans* (Table 45.1).

5 Discussion

The diagnosis of occupational HP is often made after exposure cessation, because subjects experience work-related symptoms and realize that the work environment causes them. In view of the overall favorable prognosis of HP patients are referred for a definite diagnosis weeks or months after exposure cessation symptom-free, with normal chest x-ray or computed tomography and without lung function impairment. Also in the present case the diagnosis was based on typical symptoms, lymphocytosis of bronchoalveolar lavage, the demonstration of mold-specific IgG antibodies and the disappearance of symptoms after exposure cessation. Thus the diagnosis of HP can be made with reasonable certainty and challenge testing was not considered ethical.

Although the environmental survey showed only low numbers of molds and no actinomycetes in one sump probe and no relevant concentrations of microorganisms in air samples, the results of the inhibition tests demonstrate that antigenic activity was present in MWF2, but not in MWF1. This finding indicates that monitoring of MWFs by culturing of molds may be limited by producing false-negative results that may lead to diagnostic problems because cases with occupational HP often do not show the complete spectrum of the disease due to early exposure cessation. If the medical diagnosis is not certain and environmental surveys of the workplaces do not show relevant microbial contamination, the subjects may be not diagnosed correctly. This has also consequences for the prevention of further cases in the respective industrial setting.

The present results do not allow to define a single causative mold, but they demonstrate a clear link between disease and exposure. This is especially evident for molds which could be cultivated only in small numbers as *Fusarium* sp. in the contaminated MWF, but induced strong specific IgG responses and exhibited inhibitory capacity. As no immunologic tests with bacteria were performed (with the exception of *Saccharopolyspora rectivirgula*), we cannot exclude an additional role of these microorganisms for the development of the disease. However, in view of the identification of major well-known fungal antigens in the MWF such commercially unavailable tests were not considered.

Environmental surveys during outbreaks of HP showed no (Robertson et al. 2007) or low (Tillie-Leblond et al. 2011; Fishwick et al. 2005; Hodgson et al. 2001; Fox et al. 1999) fungal contamination of sump probes. Actinomycetes were not found in any of these studies. Consequentially none of the outbreak studies in the literature considered molds or actinomycetes as the primary antigens. Although the relevant antigens could not be clearly defined in these studies, the discussion focused on mycobacteria or various gram-negative bacteria (Tillie-Leblond et al. 2011; Fishwick et al. 2005). However, in the present study molds which are among the most frequent antigens for HP in various occupational settings (Kurup et al. 2006) were identified as antigenic sources in MWFs. Although we detected specific IgG antibodies to the actinomycete *Saccharopolyspora rectivirgula* in the patient's serum, inhibition rates with this bacterium as solid phase were low. Thus we cannot precisely estimate the causal role of actinomycetes in the present case. A recent study demonstrated antibodies in sera of affected workers with exposure to MWFs directed to various mold and actinomycete antigens (Fox et al. 1999). In view of the inability to culture these microorganisms in sump probes this finding was interpreted as generalized hyperimmune response. In the present study no generalized immune response can be assumed because both direct IgG antibody binding and inhibition tests with sump proteins varied considerably between single microorganisms.

Although we recognize that this is a case study and the results may not be generalized, we recommend to include immunologic testing to molds and possibly actinomycetes in the diagnostic work-up of workers with HP and exposure to MWFs. IgG inhibition tests may help to establish the link between exposure and disease for workers with suspected HP due to MWFs, irrespective of the results of culture methods.

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

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Angiogenic Axis Angiopoietin-1 and Angiopoietin-2/Tie-2 in Non-Small Cell Lung Cancer: A Bronchoalveolar Lavage and Serum Study

46

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Abstract

Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2), ligands for the Tie-2 receptor expressed on endothelial cells, play a critical role in angiogenesis, in concert with vascular endothelial growth factor (VEGF). Angiogenesis is important for tumor growth and development and also is implicated in the pathogenesis of interstitial lung diseases. The aim of this study was to evaluate the concentration of Ang-1, Ang-2, Tie-2, interleukin-18 (IL-18), transforming growth factor beta-1 (TGF β1), and VEGF domain in both serum and bronchoalveolar lavage fluid (BALF) of lung cancer patients before chemotherapy. We studied 45 non-small cell lung cancer (NSCLC) patients (M/F; 38/7; mean age 62 ± 4 years). The age-matched control groups consisted of 15 sarcoidosis (BBS), 15 hypersensivity pneumonitis (HP), and 15 healthy subjects. The patients with NSCLC had a significantly higher level of Ang-1 compared with the BBS and healthy subjects, and a higher level of Ang-2 compared with the healthy subjects in both serum and BALF. BALF level of IL-18 was lower in the NSCLC than that in the HP group, but higher than that in the BBS patients. Serum level of IL-18 was higher in the NSCLC than in the healthy subjects. The NSCLC group had lower VEGF in BALF than that in healthy subjects. Receiver-operating characteristics (ROC) curves were applied to find the cut-off the serum levels of Ang-1 and Ang-2 levels in BALF. We did not find any correlation between the levels of Ang-1, Ang-2, Tie-2, and the stage of tumor or treatment response (prospectively). We conclude that the angiogenic axis Ang-1 and Ang-2/Tie-2

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_46,

may play an important role in lung cancer development and their concentrations may be a useful marker at the time of initial diagnosis of lung cancer.

Keywords
Angiopoietin-1 • Angiopoietin-2 • BALF • Interleukin-18 • Lung cancer • Transforming growth factor beta-1 • Tie-2 • VEGF

1 Introduction

Angiogenesis is an essential biologic event in the pathogenesis of human malignancy. Both angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) have been identified as ligands for Tie-2, a receptor expressed on endothelial cells. It has been shown that Ang-1 and Ang-2 play critical roles in angiogenesis, in concert with vascular endothelial growth factor (VEGF) (Maisonpierre et al. 1997). Ang-1 stabilizes blood vessels by promoting the interaction between endothelial cells and the surrounding extracellular matrix, and Ang-2 antagonizes the stabilizing action of Ang-1 by binding to Tie-2 competitively, which destabilizes vessels. The vessels destabilized by Ang-2 do not regress, but undergo angiogenic changes in the presence of angiogenic factors such as VEGF (Maisonpierre et al. 1997). Park et al. (2007, 2009) suggest that serum Ang-2 and Ang-1 are clinical markers of non-small cell lung cancer (NSCLC). Margaritopoulos et al. (2010) described the important role of Ang-1 and Ang-2 in patients with interstitial lung diseases.

IL-18 acts as an angiogenesis inhibitor and a tumor suppressor (Coughlin et al. 1998). IL-18 is produced by activated macrophages, dendritic cells, and Kupffer cells (Belardelli and Ferrantini 2002). Our previous investigations (Naumnik et al. 2004.) showed a higher serum level of IL-18 in lung cancer patients than in healthy persons. Liu et al. (2011) reported high concentrations of IL-18 in serum and BALF of patients with sarcoidosis.

VEGF is the most extensively studied angiogenic factor that has been related to tumor progression, metastasis, and prognosis (Han et al. 2001). VEGF is expressed in NSCLC cells and a correlation between VEGF concentration and tumor neo-angiogenesis has been documented (Han et al. 2001).

Transforming growth factor beta-1(TGF-β1) is one of the numerous inhibitory factors produced by cancer cells that regulate antitumor immunity. TGF-β1 is involved in the inhibition or activation of lymphocytes and macrophages and takes part in fibrotic processes (Wahl 1994). The findings of Domagala-Kulawik et al. (2006) confirmed that TGF-β1 takes part in a local response in the course of primary lung cancer.

In the present study, we set out to measure the serum and BALF levels of Ang-1, Ang-2, Tie-2, VEGF, IL-18, and TGF-β1 in NSCLC patients and to assess their interrelationship and clinical significance.

2 Methods

The study was performed in conformity with the Declaration of Helsinki for Human Experimentation of the World Medical Association and the protocol was approved by a local Ethics Committee. Written informed consent was obtained from all participants.

2.1 Patients and Healthy Subjects

The study involved 45 patients (38 men, mean age 62 ± 4 years) with the histological diagnosis of NSCLC (18 at clinical stage IIIB, 27 at stage IV). Squamous cell carcinoma (SCC) comprised

49 % (22 individuals) of patients with NSCLC and adenocarcinoma was revealed in 20 % (9 patients), whereas NSCLC was diagnosed in 31 % (14 patients). Serum samples obtained from the whole blood of patients with lung cancer before a cytoreduction treatment were used as the study material. Blood serum was stored at −80 °C immediately after separation by centrifugation (3,000 rpm) until the assay. At the first stage, blood samples were taken to assess Ang-1, Ang-2, Tie-2, IL-18, TGF-β1, and VEGF after complete diagnostics of lung cancer had been made, including X-ray and CT of the chest, and bronchofiberscopy with H-P test. Clinical analysis comprised the evaluation of clinical staging of NSCLC (TNM and IASLC). Chemotherapy of NSCLC patients was carried out in a 21-day cycle using cis-platin at a dose of 30 mg/m^2 on Days 1, 2, and 3, and gemcytabine at a dose of 1,000 mg/m^2 on Days 1 and 8 of the cycle. All patients received four cycles of chemotherapy. The response to therapy was estimated after four cycles of chemotherapy according to the RECIST (Response Evaluation Criteria in Solid Tumors) criteria (Therasse et al. 2000).

The reference groups consisted of 15 patients with pulmonary sarcoidosis (BBS, Besnier-Boeck-Schaumann disease) and 15 with hypersensivity pneumonitis (HP) (extrinsic allergic alveolitis), both groups were age-matched with the NSCLC. BBS patients were in the second stage of the disease, confirmed by X-ray of the chest, high resolution computed tomography of the lungs, and histological examination (Hours et al. 2008). Patients with HP were in a subacute stage, confirmed by HRCT of the chest, alveolitis in BAL, and exposure history to the known offending antigen. Bronchoscopy and BALF in all patients was performed as a part of routine clinical management. The bronchoscope was inserted and wedged in the right middle lobe, and three 50 ml aliquots of sterile saline solution, warmed to 37 °C, were instilled into the subsegmental bronchus. In patients with NSCLC, bronchoscope was inserted into the segment nearest the tumor. Fluid was gently aspirated immediately after each aliquot was introduced, and collected in a sterile container. A second 50 ml aliquot of recovered fluid was labeled as BALF. BALF samples were analyzed for total and differential cell counts, and for Ang-1, Ang-2, Tie-2, IL-18, TGF-β1, and VEGF levels detected by ELISA. One aliquot was reserved for a total cell number using Nageotte's chamber and these results were expressed as cells × 10^5/ml. The remaining fluid was immediately centrifuged at 800 rpm for 10 min at 4 °C. The differential cell profile was made under a light microscope (magnification × 1,000) by counting at least 400 cells.

The control group for serum and BALF analysis consisted of 15 healthy volunteers (13 men, mean age 60 ± 5 years) without any acute or chronic inflammatory conditions.

2.2 Serum and Ang-1, Ang-2, Tie-2, IL-18, TGF-β1, and VEGF Analysis

Ang-1, Ang-2, Tie-2, IL-18, TGF β1, and VEGF concentrations were determined by means of an enzyme-linked immunosorbent assay (ELISA, MBL-R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. All specimens were assayed twice and the average of the two measurements was used in the data analysis. The minimum detectable levels of Ang-1, Ang-2, Tie-2, IL-18, and TGF-β1 were 3.45 pg/ml, 8.29 pg/ml, 0.014 ng/ml, 12.5 ng/ml, 4.61 pg/ml, and 5 pg/ml, respectively.

2.3 Statistical Analysis

Statistical analysis was performed using Statistical 10.0 software. Data distribution was analyzed with the Shapiro Wilk test. A *t*-test for dependent or independent data was used to compare the respective pairs and groups. The Mann–Whitney U and Wilcoxon tests were used for the features inconsistent with the normal data distribution. Correlations between the parameters were calculated by the Spearman rank test. Receiver-operating characteristics (ROC) curves were applied to find the cut-off levels of Ang-1 and Ang-2. A $p < 0.05$ was considered to indicate statistical significance.

3 Results

The levels of Ang-1, Ang-2, Tie-2, IL-18, TGF-β1, and VEGF in serum and BALF are shown in Fig. 46.1. Serum Ang-1 and Ang-2 were higher in the NSCLC patients than those in the BBS patients [Ang-1: 43.01 (11.8–112.61) ng/ml vs. 24.24 (3.0–93.1) ng/ml, respectively, $p = 0.025$; Ang-2: 2.6 (0.8–9.0) ng/ml vs. 1.94 (1.0–5.0) ng/ml, respectively, $p = 0.03$]. The HP patients also had higher serum levels of Ang-1 than those in the BBS group [51.01 (20.7–109.7) ng/ml vs. 24.24 (3.0–93.1) ng/ml, respectively, $p = 0.03$]. Serum levels of Ang-2 were higher in the NSCLC group than those in both BBS and healthy subjects [2.6 (0.8–9.0) ng/ml vs. 1.94 (1.0–5.0) ng/ml vs. 1.72 (0.9–5.5) ng/ml, respectively, $p < 0.03$]. All investigated groups had similar serum concentrations of Tie-2 (Fig. 46.1a).

The NSCLC patients had higher BALF levels of Ang-1 and Ang-2 than those in the healthy subjects [Ang-1: 2.02 (2.0–120.1) ng/ml vs. 0.01 (0.0–5.2) ng/ml, respectively, $p = 0.04$; Ang-2: 62.28 (54.1–312.6) pg/ml vs. 59.57 (55.5–64.9) pg/ml, respectively, $p = 0.04$]. Ang-2 concentrations in BALF were higher in the BBS than those in the healthy subjects [61.6 (55.5–326.1) pg/ml vs. 59.57 (55.5–64.9) pg/ml, respectively, $p = 0.04$]. All investigated groups had similar concentrations of Tie-2 in BALF (Fig. 46.1b).

The NSCLC group had higher serum levels of IL-18 than healthy subjects [281.07 (118.1–1087.7) pg/ml vs. 223.77 (36.3–683) pg/ml, respectively, $p = 0.033$]. The serum

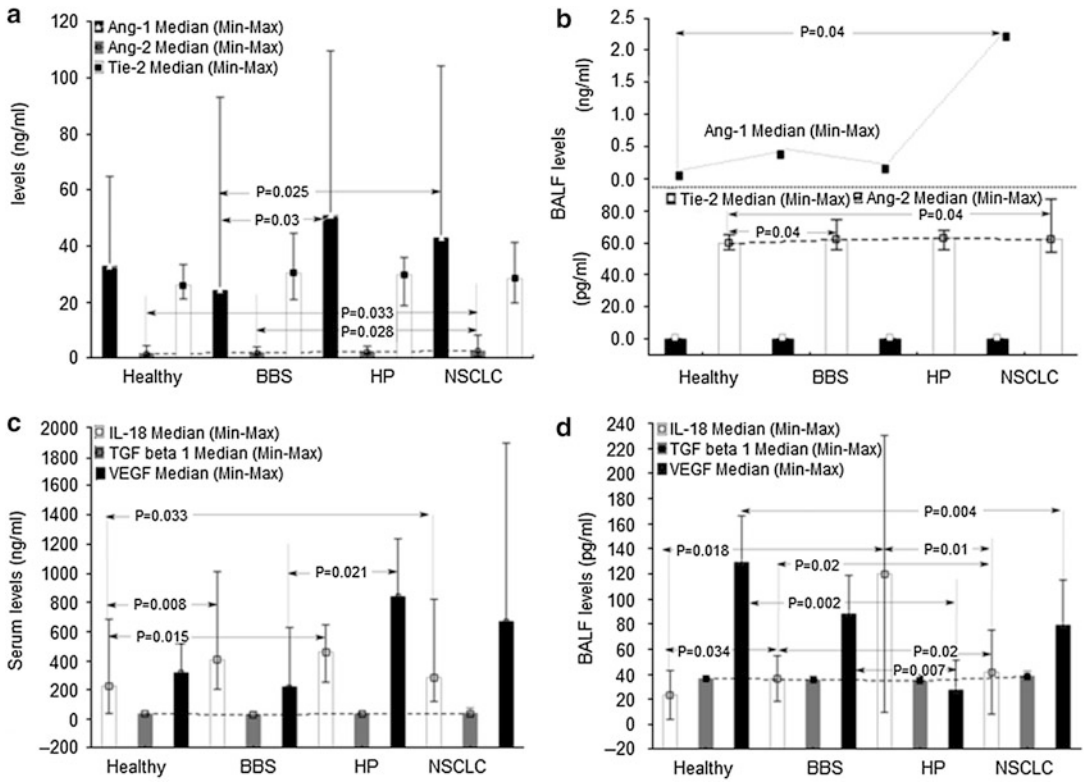

Fig. 46.1 **Comparison of angiogenic factors** in healthy people with those in patients with Besnier-Boeck-Schaumann sarcoidosis (*BBS*), hypersensivity pneumonitis (*HP*), and non-small cell lung cancer (*NSCLC*). *Panels a* and *b* – Ang-1, Ang-2, and Tie-2 in serum and in bronchoalveolar lavage fluid (*BALF*), respectively; *Panels c* and *d* – IL-18, TGF-β1, and VEGF in serum and in bronchoalveolar lavage fluid (*BALF*), respectively. Data are medians with minimum-maximum

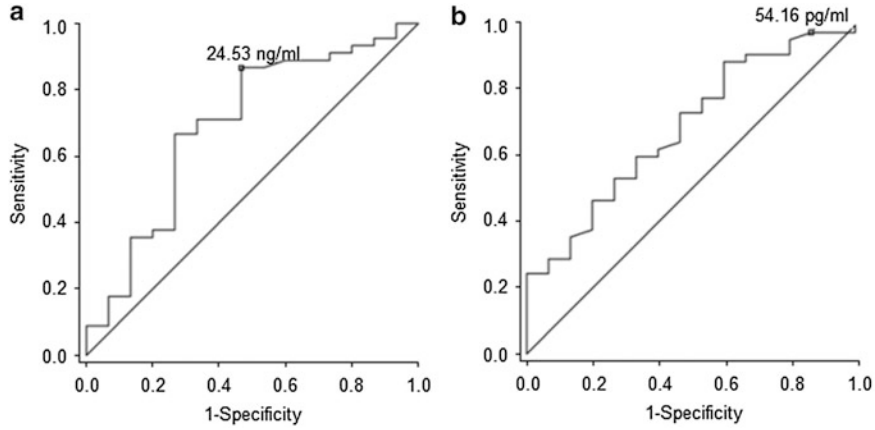

Fig. 46.2 Receiver operating characteristic (ROC) curve. (**a**) ROC curve for the serum Ang-1 in differentiating non-small cell lung cancer (NSCLC) and Besnier-Boeck-Schaumann sarcoidosis (BBS); (**b**) ROC curve for the bronchoalveolar lavage fluid (BALF) Ang-2 in differentiating non-small cell lung cancer (NSCLC) and healthy condition

concentrations of IL-18 in the healthy subjects were lower than those in the BBS and HP patients [223.77 (36.3–683) pg/ml vs. 403.56 (200.8–1,613) pg/ml vs. 456.97 (250.6–645.1) pg/ml, respectively, $p < 0.02$]. The serum levels of VEGF were higher in the HP group than those in the BBS group [839.07 (141.1–2,410) pg/ml vs. 403.56 (200.8–1,613) pg/ml, $p = 0.021$]. There were no significant differences in serum TGF-β1 levels between the groups (Fig. 46.1c)

Healthy people had higher BALF levels of VEGF than the patients with NSCLC and HP [134.1 (5.5–208.1) pg/ml vs. 46.21 (0.2–731.3) pg/ml vs. 15.77 (6.0–92.7) pg/ml, respectively, $p < 0.01$]. In the BBS group, there were higher BALF levels of VEGF than those in the HP group [79.17 (11.5–165.4) pg/ml vs. 15.77 (6.0–92.7) pg/ml, respectively, $p = 0.007$]. IL-18 levels in BALF were higher in the HP group than those in the NSCLC and healthy people [64.01 (5.7–333.7) pg/ml vs. 10.65 (2.9–733.8) pg/ml vs. 12.07 (1.9–142.1) pg/ml, respectively, $p = 0.02$]. The BBS patients had higher BALF levels of IL-18 than the healthy subjects, but lower than the NSCLC patients [28.99 (7.8–122.2) pg/ml vs. 12.07 (1.9–142.1) pg/ml vs. 10.65 (2.9–733.8) pg/ml, respectively, $p < 0.03$]. There were no significant differences in the levels of TGF-β1 in BALF between the groups (Fig. 46.1d).

ROC curves of serum Ang-1 and BALF Ang-2 concentrations were constructed to determine the cut-off values. Sensitivity and specificity of serum Ang-1 levels in the lung cancer patients relative to the BBS group were 86 and 53.0 %, respectively, at a cut-off value of 24.52 ng/ml. Sensitivity and specificity of BALF Ang-2 levels in the lung cancer patients relative to the healthy group were 98 and 86 %, respectively, at a cut-off value of 54.16 ng/ml. The area under the curve for serum Ang-1 and BALF Ang-2 were 0.694 and 0.685, respectively (Fig. 46.2).

We did not find any correlation between the levels of Ang-1, Ang-2, Tie-2, and TGF-β1 and the stage of tumor or the response to treatment. The BALF levels of IL-18 were higher in Group IIIB than those in Group IV NSCLC patients [14.21 (2.9–176) pg/ml vs. 7.48 (2.9–733) pg/ml, $p = 0.032$]. Patients with progressive disease after treatment (prospectively) had higher serum levels of VEGF than those with partial response [960 (122.1–2,077) pg/ml vs. 477.05 (98–1,414) pg/ml, $p = 0.034$].

In the NSCLC group, a positive correlation was found between the BALF levels of Tie-2 and

Ang-1 ($r = 0.412$, $p = 0.004$), BALF Tie-2 and Ang-2 ($r = 0.424$, $p = 0.003$), BALF VEGF and Ang-1 ($r = 0.357$, $p = 0.017$), BALF IL-18 and Ang-1 ($r = 0.351$, $p = 0.018$). We also observed a positive correlation between the levels of IL-18 and lymphocyte count in BALF ($r = 0.343$, $p = 0.02$). In the BBS group, we found the following correlations: between serum IL-18 and ACE (angiotensin-converting enzyme) ($r = 0.550$, $p = 0.001$), serum Tie-2 and ACE ($r = 0.556$, $p = 0.004$), BALF Ang-2 and serum VEGF ($r = 0.689$, $p = 0.002$), BALF Tie-2 and serum VEGF ($r = 0.734$, $p = 0.001$), BALF IL-18 and serum VEGF ($r = 0.684$, $p = 0.004$), and BALF Ang-2 and IL-18 ($r = 0.683$, $p = 0.003$).

4 Discussion

In this study, we evaluated the concentrations of Ang-1, Ang-2 and Tie-2 in bronchoalveolar lavage fluid and serum of patients with advanced NSCLC. The reference groups consisted of from patients with Besnier-Boeck-Schaumann disease (BBS), hypersensitivity pneumonitis (HP), and healthy persons. Recently other researchers have reported on the role of angiogenesis in patients with BBS and HP (Zielonka et al. 2007; Cui et al. 2010). Angiogenesis-angiostasis balance may contribute to the different immunopathogenesis, clinical course and responsiveness to treatment of these diseases (Cui et al. 2010). We also analyzed the levels of IL-18, TGF-β1, and VEGF, which all have been reported previously as disease modifiers in patients with lung cancer and interstitial lung diseases (Takai et al. 2012; Liu et al. 2011). In this study we confirmed the presence of interactions between these molecules in the disease process. We found that patients with NSCLC had higher serum levels of Ang-1 than the BBS group. The highest serum level of this molecule was observed in HP patients. The level of Ang-1 in BALF was higher in the NSCLC than that healthy subjects. Our study is consistent with the findings of Ye et al. (2009) and Tait and Jones (2004). There are different reasons for the increase in Ang-1 in NSCLC and HP patients. Ang-1 has a dual contrasting function. In normal tissues, during an inflammatory reaction mediated by IL-18, Ang-1 promotes angiogenesis in HP patients (Park et al. 2009; Ye et al. 2009). We confirmed this findings because HP patients had a high level of VEGF in the serum and a high level of IL-18 in BALF.

Ang-1 acts as a natural antagonist of Ang-2 by binding to Tie-2 competitively and in the presence of VEGF destabilizes blood vessels in cancer patients (Maisonpierre et al. 1997). However, when this equilibrium is disturbed, e.g., by the absence of VEGF, Ang-1 promotes neoangiogenesis in mice (Shim et al. 2002). These findings are in conformity with our study. The NSCLC patients had a lower level of VEGF in BALF than healthy subjects. Our observations are different from a study of Charpidou et al. (2011) who found a higher level of VEGF in NSCLC patients. Furthermore, Varet et al. (2010) reported on the novel isoforms of VEGF165b in the alveolar space which may have an inhibitory role in pulmonary angiogenesis, and which may contribute to discrepant results.

Increased angiogenesis occurs in the lungs of patients with BBS and HP (Zielonka et al. 2007; Sekiya et al. 2003). Our present results are in accord with those of Sekiya et al. (2003). We observed a high level of Ang-2 in BALF from HP and BBS patients. Moreover, there were high levels of VEGF and IL-18 in BALF from HP patients. A high level of IL-18 in HP is due likely to granulomatous inflammation and release of this molecule from alveolar macrophages. We found a positive correlation between the level of IL-18 in BALF and the macrophage count, which confirms the findings of Ye et al. (2009).

The level of IL-18 was higher in BALF from the NSCLC group than that from the healthy group. We observed a correlation between the level of IL-18 and the lymphocyte count in BALF. Probably, IL-18 was released from alveolar lymphocytes. These findings are in accord with those from other studies as well (Vidal-Vanaclocha et al. 2006).

Finally, we constructed the ROC curves for the serum Ang-1 and the BALF Ang-2 to

determine the cut-off values. Sensitivity and specificity of BALF Ang-2 level in lung cancer patients relative to the control group were 98 and 86 %, respectively. Other authors have described a lower values for sensitivity and specificity of Ang-2 in the serum: 80 and 55 %, respectively (Park et al. 2007). Although the serum Ang-2 is a potentially useful adjunct for diagnosis, it appears insufficient as a single diagnostic marker for lung cancer.

TGF-β1 takes part in a local response in the course of primary lung cancer (Domagala-Kulawik et al. 2006) and fibrotic processes (Wahl 1994). However, in the present study, we did not find any clinical usefulness of TGF-β1 at the time of lung cancer diagnosis.

Summing up, the angiogenic axis Ang-1 and −2/Tie-2 may play an important role in lung cancer development and their concentrations may be useful at the time of initial diagnosis of lung cancer. Moreover, the angiogenic axis may also play a role in common interstitial lung diseases, such as sarcoidosis and hypersensivity pneumonitis.

Acknowledgments We are indebted to Robert Mroz for his help with the ELISA equipment. Supported by granted from the Polish Ministry of Science and Higher Education (NN 401 230134; 2301/B/P01/2008/34).

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

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Cancer Incidence in Pulmonary Vasculitis

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Abstract

Pulmonary vasculitis is a potentially lethal autoimmune disease characterized by granulomatous inflammation of respiratory tract, necrotizing vasculitis affecting small-to medium-size vessels and antineutrophil cytoplasmic antibodies elevation. Typical therapy involves high-dose glucocorticosteroids combined with cyclophosphamide in a dose 1–2 mg/kg/per day. A high relapse rate in pulmonary vasculitis means prolonged courses of cyclophosphamide in some patients. Carcinogenic effects of cyclophosphamide, especially its toxic metabolite acrolein that is excreted into the urine, are responsible for the development of acute myeloid leukemia (AML) and bladder cancer. These and other malignancies are cyclophosphamide dose-depended. The aim of the present study was to assess the incidence of cancer in patients with pulmonary vasculitis in comparison with the incidence of cancer in the general population. Analyses were done according to the cumulative dose of cyclophosphamide, subdivided into low ($\leq$35 g) and high ($>$35 g). During the observation period 15 cancers occurred. A significantly increased standardized incidence ratio (SIR) was observed for non-melanoma skin cancers (SIR 5.2; 95 % Cl 2.3–8.7), AML (SIR 4.3; 95 % Cl 2.1–11.2), and bladder cancer (SIR 3.4; 95 % Cl 1.6–5.2). Induction remission treatment and relapse treatment with cyclophosphamide involves a substantial risk of late appearing malignances in patients with pulmonary vasculitis. Monitoring and prophylactic management in pulmonary vasculitis after cessation of cyclophosphamide therapy is crucial.

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_47,

Keywords
Acrolein • Antineutrophil cytoplasmic antibodies • Autoimmune disease • Cancer • Cyclophosphamide • Pulmonary vasculitis • Risk

1 Introduction

Pulmonary vasculitis is a potentially lethal autoimmune disease characterized by granulomatous inflammation of respiratory tract, necrotizing vasculitis affecting small-to medium-size vessels, and an elevation of antineutrophil cytoplasmic antibodies against proteinzase-3, myeloperoxidase, or both. Typical therapy for remission induction involves high-dose glycocorticosteroids in combination with cyclophosphamide in a dose 1–2 mg/kg per day. A high relapse rate in pulmonary vasculitis means prolonged courses of cyclophosphamide in some patients. Cyclophosphamide is a nitrogen mustard alkylating agent from the oxazophorine group. It is used to treat various types of cancer and some autoimmune disorders. Cyclophosphamide is converted by mixed-function oxidase enzymes in the liver to active metabolites. The main active metabolites are 4-hydroxycyclophosphamide and its tautomer, aldophosphamide. Most of the aldophosphamide is oxidized by the enzyme aldehyde dehydrogenase to carboxyphosphamide. Aldophosphamide is converted into phosphoramide mustard and acrolein (Colvin 1999). Carcinogenic effects of cyclophosphamide, especially of its toxic metabolite acrolein that is excreted into the urine, are responsible for a high risk of hemorrhagic cystitis, acute myeloid leukemia (AML), and bladder cancer. These and other malignancies are cyclophosphamide dose-depended (Bernatsky et al. 2008; Molloy and Langford 2008). The risk of carcinogenesis depends on the dose of cyclophosphamide, but also on a number of other factors, including the clinical status of a patient, hydration, other agents, or treatment modalities used, foremost radiotherapy, and on treatment intensity and length.

Early studies assessing the risk of malignancy in pulmonary vasculitis have mainly focused on cyclophosphamide related bladder cancers. The risk of this malignancy has been found not to increase in patients who never receive cytotoxic drugs. A dose-depended increase in risk of cancers has also been reported for cyclophosphamide-treated patients with rheumatoid arthritis (RA). Furthermore, several other chronic autoimmune diseases such as systemic lupus erythematosus (SLE), inflammatory bowel disease, Sjögren's syndrome, sarcoidosis, primarily sclerosing cholangitis, or celiac disease are also associated with increased occurrence of cancers, such as lymphomas, non-Hodgkin's lymphomas, colorectal cancer, small intestine carcinomas, or cholangiocarcinomas. There have been reports of highly increased risk for cancer of the urinary bladder in patients with non-Hodgkin's lymphomas and Wegener's granulomatosis, but the groups studied were small (Le Guenno et al. 2011; Knight et al. 2002). The aim of the present study was to assess the incidence of cancer in a population of Polish patients suffering from pulmonary vasculitis in comparison with that in the general population.

2 Methods

The study was approved by a local Ethics Committee. The discharge logs of the Primary Vascular Outpatient Clinic of the Czerniakowski Hospital in Warsaw, Poland were used to identify patients with pulmonary vasculitis treated in the period of 1990–2008. A hundred and seventeen patients with biopsy-proven vasculitis were enrolled the study. The median age of the patients at the time of pulmonary vasculitis diagnosis was 64.5 years (range 18–88 years) and the median duration of follow-up was 7.0 years (range 0–25 years). Patients' characteristics are summarized in Table 47.1.

Table 47.1 Characteristics of the Wegener's granulomatosis group (n = 117)

	n (%)
Sex (M/F)	78/39 (67/33)
Age at diagnosis 15–19 year	3 (2.5)
Age at diagnosis 20–39 year	6 (5.1)
Age at diagnosis 40–59 year	37 (31.7)
Age at diagnosis 60–79 year	62 (52.9)
Age at diagnosis ≥80 year	9 (7.8)
Years of diagnosis 1990–1994	32 (27.3)
Years of diagnosis 1995–1999	38 (32.5)
Years of diagnosis 2000–2004	31 (26.5)
Years of diagnosis 2005–2008	16 (13.7)
CYC dose 1–35 g	91 (77.7)
CYC dose >35 g	26 (22.3)

CYC cyclophosphamide

All patients fulfilled the American College of Rheumatology criteria for classification of vasculitis and the Chapel Hill Consensus Conference definition (Sharma et al. 2011). Patients with incomplete or missing medical dates were excluded from further analysis. Disease activity was confirmed by clinical scoring, laboratory variables and typical imaging procedures. The data collected included age, sex, age at diagnosis, history of disease, year of diagnosis, disease duration, and cumulative cyclophosphamide treatment dose. A total of 90 patients received oral cyclophosphamide therapy in a dose 1–2 mg/kg per day with oral prednisolone (1 mg/kg per day). Twenty patients received cyclophosphamide as intravenous pulses of 0.75 g/m^2 per month with methylprednisolone (3 days *a* 500 mg/day). Seven patients were treated with both intravenous and oral cyclophosphamide (1–2 mg/kg/day). Azathioprine (AZA) (2 mg/kg/day) and methotrexate (MTX) (25 mg/week) were used in two cases as maintenance therapy after the achievement of a complete remission.

The population cohort studied was registered in the files of the Polish Cancer Registry (www.euro.who.int/hfadb). Malignancies were coded according to the International Classification of Disease (www.euro.who.int/hfadb). As a measure of relative risk we calculated standardized incidence ratio (SIR) of cancer. The SIR is an estimate of cancer occurrence; the ratio between observed and expected numbers of cancers. We analyzed the SIR according to sex, time of follow-up, and age at cancer diagnosis. Ninety-five percent confidence intervals (95 % Cl) assumed a Poisson discrete probability distribution of the cases observed.

3 Results

We report the occurrence of 15 cancers: 6 skin non-melanomas (1 planoepithelial cancer, 1 squamous cell carcinoma, and 4 basal cell carcinomas), 3 bladder cancers, 1 colorectal cancer, 2 lower respiratory tract cancers, 2 acute myeloid leukemias, and 1 cancer of male genitals. Significantly increased SIRs were observed for skin cancer (SIR 5.2; 95 % Cl 2.3–8.7), acute myeloid leukemia (AML) (SIR 4.3; 95 % Cl 2.1–11.2), and for bladder cancer (SIR 3.4; 95 % Cl 1.6–5.2). The SIR for different cancer types are presented in Table 47.2.

The non-melanoma skin cancers reported occurred in the sun-exposed areas, without skin protection and more often in the scared or burned skin, typically in the facial region (n = 4) and on lower extremities (n = 2) (SIR for basal cell carcinomas = 4.8; 95 % Cl 1.8–8.9; SIR for squamous cell carcinomas = 16.7; 95 % CI 5.6–38.0). Bladder cancers occurred after a median latency period of 10.4 years (range 5.7–15.4 years, n = 2). The risk for hematopoetic cancers was most pronounced in the first years of the course of pulmonary vasculities. There was no significant difference between the cumulative cyclophosphamide dose received by the patients treated with cyclophosphamide and corticosteroids (median cumulative dose of cyclophosphamide = 35 g, range 1.8–160.0 g) and the cumulative dose of cyclophosphamide received by the patients treated with cyclophosphamide plus AZA or MTX (median cumulative dose 22.0 g, range 0.9–189.0 g).

Table 47.2 Standardized incidence cancer ratio (SIR)

Cancer	Number of cancer cases	SIR (95 % Cl)
All sites	15	2.5 (1.2–2.9)
Acute myeloid leukemia	2	4.3 (2.1–11.2)
Bladder cancer	3	3.4 (1.6–5.2)
Non-melanoma skin cancer	6	5.2 (2.3–8.7)
Lung cancer	2	1.7 (0.5–3.4)
Colorectal cancer	1	1.3 (0.2–3.6)
Male genital cancer	1	2.2 (0.6–4.2)

4 Discussion

Adverse drug reactions after cyclophosphamide therapy include nausea, vomiting, bone marrow suppression, alopecia, and lethargy. Hemorrhagic cystitis is a frequent complication, but it can be prevented by optimal fluid intake and mesna (sodium 2-mercaptoethane sulfonate as a sulfhydryl donor binding acrolein). A more dangerous adverse drug effect is carcinogenesis.

In the present study we observed a strongly increased risk of cancer; the risk being most pronounced for bladder, non-melanoma skin cancer, and acute myeloid leukemia. The carcinogenic effects of cyclophosphamide, especially its toxic metabolite acrolein that is excreted into the urine, are likely responsible for a high risk of AML and bladder cancer. The risk of secondary malignancies is dose-dependent (Faurschou et al. 2008, 2009; Hoff and Rødevand 2005). It has been demonstrated that the risk of bladder cancer is associated with both dose and duration of treatment with cyclophosphamide (Talar-Williams et al. 1996). Cyclophosphamide has relatively small toxicity if aldehyde dehydrogenases (ALDHs) are present in relatively large concentration in bone marrow stem cells, hepatocytes, and epithelium. ALDHs protect the stem cells against toxic effects of phosphoramide mustard and acrolein by converting aldophosphamide to carboxyphosphamide without an elevation in acrolein. A study by Heijl et al. (2011) has demonstrated a high risk of AML (SIR = 59.0, 95 % Cl 12–72) and of bladder cancer (SIR = 9.5, 95 % Cl 2.6–24) in patients treated with a cumulative dose of cyclophosphamide >36 g. In comparison, the present study demonstrated a high relative risk of serious malignancies for patients treated with a cumulative dose of cyclophosphamide >35 g. We observed significantly increased SIRs for non-melanoma skin cancer (SIR = 5.2; 95 % Cl 2.3–8.7), AML (SIR = 4.3; 95 % Cl 2.1–11.2), and bladder cancer (SIR = 3.4; 95 % Cl 1.6–5.2). Cyclophosphamide-inducted AML typically is manifest some years after treatment, with the highest incidence around 3–9 years, and after 9 years the risk abates to the level of that in the general population. AML often occurs as myelodysplastic syndrome that transforms into acute leukemia with worse prognosis than AML *de novo*. Among non-melanoma skin cancers, there were squamous cell and basal cell carcinomas found, which occurred in the sun-exposed areas, scared or burned skin areas, typically in the facial region (SIR for basal cell carcinomas = 4.8; 95 % Cl 1.8–8.9; SIR for squamous cell carcinomas = 16.7; 95 % 5.6–38.0). The risk of non-melanoma skin cancer is usually increased from 1 year after the diagnosis of vasculitis and remains significantly increased throughout the follow-up (Faurschou et al. 2008). No case of skin cancer was observed during the first year of observation in the present study. A shorter latency between the institution of treatment and the occurrence of malignancies is strongly associated with immunosuppressive treatment (Silva et al. 2011; Takala et al. 2010; Stone et al. 2006; Pankhurst et al. 2004).

The risk of developing leukemia significantly increases between 5 and 10 years of the follow-up observation. Patients with pulmonary vasculitis should undergo the long-term follow-up with

blood and urine analyses after cyclophosphamide therapy, and should undergo a comprehensive hematologic, dermatologic, and urologic evaluation in case of abnormalities. It is recommended that all patients treated with cyclophosphamide undergo urine analysis and urine cytology every 6–12 months, even when basic therapy is finished. Any hematuria, atypia, or dysplasia should be followed up with the ultrasound examination of the abdomen, urinary tract, and with a cytoscopic evaluation. Cyclophosphamide inducted cystitis puts patients at higher risk for bladder cancer. Furthermore, there is an urgent need for effective creation of an alternative to the toxic cyclophosphamide treatment in pulmonary vasculitis and in other systemic autoimmune diseases.

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

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Influence of Delays in Diagnosis and Treatment on Survival in Small Cell Lung Cancer Patients

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Abstract

The purpose of this study was to evaluate the influence on survival of delays in the diagnosis and treatment in an unselected population of small cell lung (SCLC) patients. Demographic and disease data of 3,479 SCLC patients were registered in the National Tuberculosis and Lung Diseases Research Institute in Warsaw, Poland during 1995–1998. In 50 % of patients, treatment started within 78 days from the appearance of first symptom(s). The median delay was 30 days (mean 47 days) and the median referral delay to a specialist was 19 days (mean 36 days). Half of SCLC patients were diagnosed during 34 days (mean 55 days). The mean time elapse from the diagnosis to the onset of therapy was 30 days (median 6 days). The multivariate analysis revealed that male gender-HR (hazard ratio = 1.2), ECOG Performance Status of 2 (HR = 1.5) and 3 + 4 (HR = 2.4), and clinical stage III (HR = 1.3) and IV (HR = 1.9) of the disease were independent negative predictors of survival. The patients treated with surgery and combined modality treatment had a better prognosis than those treated with chemoradiotherapy (HR = 1.6), chemotherapy (HR = 2.5), symptomatically (HR = 4.0), or those who refused therapy (HR = 3.9). The delay in the diagnosis and treatment had no effect on survival. Interestingly, patients who were diagnosed faster (below 42 days) actually had a worse prognosis than those diagnosed later. We conclude that a prolonged workup of SCLC patients and an extended

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_48,

time for treatment onset have a positive influence on survival, which may likely have to do with the determination of disease stage and more targeted treatment.

Keywords
Diagnosis delay • Hazard ratio • Performance status • Prognosis • Small cell lung cancer • Survival • Therapy

1 Introduction

In many European countries and the US lung cancer is a leading cause of death (Siegel et al. 2012). The overall 5-year survival for patients with lung cancer is very low and varies between 18 % in the US, 8 % in Poland, to 6 % in Denmark (Tyczynski et al. 2004). Small cell lung cancer (SCLC) is one of the three most often found histological types of lung cancer. In Poland, diagnosis of SCLC is established in about 19 % of patients (27 % in women and 20 % in men) (Radzikowska et al. 2002). Five-year survival in this histological type of lung cancer is extremely low, less than 4 % (Senkus et al. 1994; Albain et al. 1990). It is well known that SCLC patients with limited disease, and in a good Eastern Cooperative Oncology Group Performance Status (ECOG PS), who achieve complete regression, have a better prognosis than those with extensive disease and bad ECOG PS. It has been shown that patients with SCLC at T1 N0M0 stage of disease, treated with combined modality therapy (surgery, chemo-, and radiotherapy) have the same prognosis as patients with NSCLC at similarly advanced stage (Chen et al. 2010). However, such studies are limited (Tamura et al. 1998; Lassen et al. 1995). Recently, data collected in the IASLC Lung Cancer Staging Project provided the documentation of 13,032 patients with SCLC (Shepherd et al. 2007). Over 8,000 of them were classified according to the TNM staging system. The fraction of patients who survived 5 years with tumor cT1, cT2, cT3, cT4 was 29, 15, 11, and 10 %, respectively, but only 1 % of patients with M1 disease survived 5 years. SCLC proliferates quickly, so fast diagnosis and immediate administration of treatment can influence survival.

The British Thoracic Society with the Joint Council for Oncology and ACCP guidelines recommended a referral policy for patients with lung cancer suspicion (Spiro et al. 2007; Buccheri and Ferrigno 2004; The National Lung Cancer Screening Research Teem (2011); BTS 1998). It is advised that GP should refer such patients for a specialist diagnostic workup within 7–14 days. The time from diagnosis to onset of treatment should not be longer than 31–42 days, and that to administration of non-surgical treatment should not exceed 7–28 days.

There are studies that provide documentation for the management process and outcome in lung cancer patients, but these studies mainly focus on patients with NSCLC and particularly those who are surgically treated (Skaugh et al. 2011; Gonzalez-Barcala et al. 2010; Olsson et al. 2009; Radzikowska et al. 2001). However, there apparently are no data that consider the influence of patients and doctors' delays in the medical approach and workup on the prognosis of SCLC patients. It was therefore the aim of the present study to perform this kind of analysis in regard to patients' survival in an unselected group of SCLC patients.

2 Methods

The study was approved by a local Ethics Committee. During the years 1995–1998, data on 3,479 SCLC patients admitted to the Pulmonary Outpatients Departments from all parts of Poland were collected in the register of National Institute

of Tuberculosis and Lung Diseases in Warsaw, Poland. Data regarding gender, age, histological type of lung cancer performance status (PS) according to ECOG scale (Oken et al. 1982), stage of the disease (TNM scale), treatment, 5-year survival, delays caused patients and doctors' inactivity were recorded using a standardized questionnaire. The dates of the first contact with a specialist doctor and of first bronchoscopy were noted for the years 1996–1998. Follow-up information was gathered every 6 months. The survival of patients was finally assessed at the end of 2003, taking into account the time period between diagnosis and death. Diagnosis of SCLC was based on positive histological and/or cytological examinations according to the WHO criteria (1981). The extent of the disease was evaluated according to the TNM classification (International Union Against Cancer 1997), but the terms limited or disseminated disease was used in the analysis.

The patient's delay was defined as a time between the appearances of first symptoms until the first visit to a doctor's office. The doctor's delay was defined as a time between the first visit of a patient until the commencement of treatment. The therapy delay was defined as a time between the date of diagnosis and that of treatment commencement. Also, the time delays between the first visit to a doctor's office until the first contact with a specialist and between the first visit to a specialist and bronchoscopy, diagnosis, and therapy were recorded.

To assess the influence of patient and doctors' delays on survival, the value of median in days was chosen. It was assumed that the patients who had a visit to GP within 30 days from symptom (s) onset were regarded as those without a delay. Likewise, the patients who were diagnosed during the first 42 days from the first visit to a doctor were regarded as those without a delay.

Data were stratified according to prognostic factors such as gender, age, PS, clinical stage of disease, and type of treatment for comparisons. Differences were tested with the Chi^2 and Pearson's tests. Univariate and multivariate analysis by Cox proportional hazards ratio (HR) model and a log-rank test were used to test the significance of the prognostic factors above outlined. A $p < 0.05$ was considered to be significant.

3 Results

Altogether 620 women and 2,859 men with SCLC were registered. The characteristics of patients were presented in Table 48.1. The mean age of all patients was 60 ± 10 years.

Table 48.1 Characteristics of SCLC patients

	All	Women	Men	p
Age	60 ± 10	58 ± 10	60 ± 10	0.001
ECOG PS				
0 + 1	386 (12.2 %)	320 (12.4 %)	66 (11.7 %)	NS
2	1,003 (31.8 %)	817 (31.6 %)	186 (32.9 %)	
3 + 4	1,756 (56 %)	1,444 (56 %)	312 (55.4 %)	
Clinical stage				
LD	841 (31.6 %)	693 (32.2 %)	148 (30.9 %)	NS
ED	1,818 (68.4 %)	1,491 (67.8 %)	328 (69.1 %)	
Treatment				
Chemotherapy	1,689 (64.8 %)	1,404 (65.2 %)	285 (62.6 %)	NS
Chemoradiotherapy	362 (13.9 %)	282 (13.1 %)	80 (17.6 %)	0.001
Surgery + chemo- and/or radiotherapy	150 (5.7 %)	115 (5.3 %)	35 (7.6 %)	0.05
Supportive care	342 (13.1 %)	296 (13.7 %)	46 (10 %)	0.05
Refused	67 (2.5 %)	58 (2.7 %)	9 (2 %)	NS

ECOG PS Eastern Cooperative Oncology Group Performance Status, *LD* limited-stage disease, *ED* extensive-stage disease, *NS* non-significant

Table 48.2 Delays in the management of patients (days)

	No. of patients	Mean ± SD	Median	Low quartile	High quartile
Patient's delay	2,497	47.4 ± 80.3	30	9	59
First visit to the doctor – diagnosis	3,076	55.5 ± 78.8	34	19	61
Doctors' delay – first visit to the doctor – start of treatment	2,259	72.4 ± 100	42	15	84
First visit to the doctor – first visit to a specialist	1,652	36.3 ± 59	19.5	9	38
First visit to the specialist – bronchoscopy	1,724	20.5 ± 42	10	6	21
First visit to the specialist – diagnosis	1,907	35.3 ± 55	21	14	37
Diagnosis – treatment	2,443	28.9 ± 64.4	6	6	21
Symptom(s) – diagnosis	3,165	94.5 ± 101.1	69	41	111
Symptom(s) – therapy	2,214	113.3 ± 118.5	78	45	135

Women were significantly younger than men (58 ± 10 vs. 60 ± 10 years, respectively; $p < 0.001$). There were no gender differences in ECOG PS and the clinical stage of disease. Women were treated more frequently than men with surgery combined with chemoradiotherapy (7.6 vs. 5.3 %, respectively; $p = 0.05$) or with chemoradiotherapy (17.6 vs.13.1 %; $p = 0.01$). Also, a significantly smaller percentage of women received symptomatic treatment (10 vs. 13.7 %, respectively; $p = 0.05$).

Overall, 1-year survival was 36 %. Only 4 and 0.01 % of patients survived 2 and 5 years, respectively. Fifty percent of patients died within 281 days of the diagnosis. The delays observed at each stage of the diagnostic process are detailed in Table 48.2. The median time of total delay (from the first symptoms to the start of treatment) was 78 days (mean 113.3 ± 118.5 days). The median patient's delay was 30 days (mean 47.4 ± 80.3 days). The median time between the first visit to the GP and diagnosis was 34 days (mean 55.5 ± 78.8 days). Within the first 6 days from diagnosis, treatment was administered to 50 % of the patients (mean 28.9 ± 64.4 days). The median referral delay was 19.5 days (mean 36.3 ± 59 days). The specialists established the diagnosis in a median time of 21 days (mean 35.3 ± 55 days). Also, 50 % of the patients had bronchoscopy within 10 days (mean 20.5 ± 42 days). The median time from the first contact with a doctor and onset of therapy was 42 days (mean 72.4 ± 100). It should be underlined that, although there were many cases without any delays, in other cases, despite a long symptomatic period, diagnosis had not been established for several months.

The univariate analysis of factors which can influence survival revealed that male gender (HR = 1.17), ECOG PS 2 (HR = 1.52) and 3 + 4 (HR = 2.42), and clinical stages of disease III (HR = 1.32) and IV (HR = 1.89) had a significant negative impact on survival. The patients treated with surgery and chemoradiotherapy (HR = 1.00) had a better prognosis than those who received chemoradiotherapy (HR = 1.59), chemotherapy (HR = 2.52), or were treated symptomatically (HR = 4.0) (Table 48.3).

The multivariate analysis of absolute survival revealed that ECOG PS 2 (HR = 1.51) and 3 + 4 (HR = 2.41), clinical stages of disease III (HR = 1.22) and IV (HR = 1.61), chemoradiotherapy (HR = 1.6), chemotherapy (HR = 2.44), and the lack of doctors' delay (HR = 1.20) were negative predictors of survival in SCLC patients (Table 48.4).

The ECOG PS at the initial presentation was one of the most powerful prognostic variables identified in the present study. Therefore, multivariate analysis of the factors above outlined was performed on the patients stratified according to ECOG PS (Table 48.5). Female sex was found as a factor that conferred survival benefit, but only in

Table 48.3 Hazard ratios (HR) for death in small cell lung cancer patients – univariate analysis

	HR	p
Gender		
Women	1.00	
Men	1.17	0.016
Treatment		
Combined + surgical	1.00	
Chemoradiotherapy	1.59	0.003
Chemotherapy	2.52	<0.001
Symptomatic	4.00	<0.001
Refused	3.85	<0.001
ECOG PS		
0 + 1	1.00	
2	1.52	<0.001
3 + 4	2.42	<0.001
Clinical stage		
LD	1.32	
ED	1.89	<0.001
Patient's delay		
Shorter than median time (30 days)	1.00	
Longer than median time (30 days)	0.91	NS
Doctor's delay		
Shorter than median time (42 days)	1.20	0.001
Longer than median time (42 days)	1.00	

ECOG PS Eastern Cooperative Oncology Group Performance Status, *LD* limited-stage disease, *ED* extensive-stage disease, *NS* non-significant

Table 48.4 Hazard ratios (HR) for death in small cell lung cancer patients – multivariate analysis

	HR	p
Gender		
Women	1.00	
Men	1.09	0.221
Treatment		
Combined + surgical	1.00	
Chemoradiotherapy	1.60	0.002
Chemotherapy	2.44	<0.001
ECOG PS		
0 + 1	1.00	
2	1.51	<0.001
3 + 4	2.41	<0.001
Clinical stage		
LD	1.22	
ED	1.61	<0.001
Patient's delay		
Shorter than median time (30 days)	1.00	
Longer than median time (30 days)	0.95	NS
Doctors' delay		
Shorter than median time (42 days)	1.20	
Longer than median time (42 days)	1.00	0.002

ECOG PS Eastern Cooperative Oncology Group Performance Status, *LD* limited-stage disease, *ED* extensive-stage disease, *NS* non-significant

the group of patients in good ECOG PS. Despite differences in ECOG PS, the patient's delay had no influence on survival in none of the patients' groups. On the other hand, an influence of the doctor's delay on survival was observed in all patients, but it assumed significance only in the patients in ether very good or bad condition. The patients in ECOG PS 0 + 1 or 3 + 4, who had been diagnosed for the longer periods of time, had a better prognosis than those diagnosed faster.

4 Discussion

In the present study, 1-year survival of SCLC patients was 36 %, and only 4 % of patients survived 2 years. This survival rate is slightly lower than that reported in other studies concerning the patients enrolled to clinical trials or treated in oncological centers, but it is similar to that reported by Laskin et al. (2004) in the population-based data. The prognosis of patients with SCLC is overall extremely bad (Shepherd et al. 2007). Senkus et al. (1994), in a Polish population of SCLC patients, diagnosed and treated in two medical centers, showed that 10 % of patients survive 2 years and 3.8 % survive 5 years. In a Danish study, Lassen et al. (1995) observed a 3.5 % 5-year survival among 1,714 unselected SCLC patients. Analysis of 2,580 patients enrolled to the Southwest Oncology Group clinical trials, revealed that 23 % of patients with extensive disease survive 1 year, and 24 % of patients with limited disease survive 2 years, but only 4 % of patients with extensive disease survive 2 years (Albain et al. 1990). Chen et al. (2010) published data regarding the improved survival of SCLC patients with limited disease. The authors pointed out that, particularly in this group of patients, early (within 30 days from diagnosis) chemotherapy and radiotherapy, and prophylactic cranial irradiation provide a

Table 48.5 Hazard ratios (HR) for death in small cell lung cancer patients – multivariate analysis according to ECOG PS

	ECOG PS					
	0 + 1		2		3 + 4	
	HR	p	HR	p	HR	p
Gender						
Women	1.0		1.0		1.0	
Men	1.23	0.03	1.08	NS	0.97	NS
Age						
Below 50 years	1.0		1.0		1.0	
Over 50 years	0.74	0.014	1.08	NS	1.44	0.05
Clinical stage						
LD	1.0		1.0		1.0	
ED	1.4	<0.001	1.8	0.001	2.1	0.02
Patient's delay						
Shorter than median time (30 days)	1.0		1.0		1.0	
Longer than median time (30 days)	0.9	NS	0.9	NS	1.2	NS
Doctors' delay						
Shorter than median time (42 days)	1.0		1.0		1.0	
Longer than median time (42 days)	1.16	0.05	1.16	NS	1.42	0.04

ECOG PS Eastern Cooperative Oncology Group Performance Status, *LD* limited-stage disease, *ED* extensive-stage disease, *NS* non-significant

significant survival advantage. In the studies above outlined as well as in the present study, ECOG PS and the type of treatment are factors influencing the survival time. Gender seems a rather weak variable influencing survival (Radzikowska et al. 2002). In the present study we found that female gender is a favorable predictor, particularly in patients in good ECOG PS. Other studies demonstrated that younger women with limited SCLC have a better prognosis than men (Albain et al. 1990).

The referral policy advised by BTS (1998) is that patients with clinical evidence of lung cancer should be immediately referred to the specialist, the diagnostic period should not be longer than 2 weeks, and that no longer than a maximum of 4 weeks the patient should wait for therapy other than surgery. In the present study, SCLC patients encounter a median delay between the first symptom(s) and treatment of 78 days. However, there were some patients who experienced symptoms or waited for the diagnosis for a longer period as the mean time delay was 4 months. It seems rather long from the perspective of highly progressive disease. Forty percent of all delay was due to the patients, another 40 % was due to diagnostic procedures, the remaining 20 % was connected with administration of therapy. In the literature, the patient's delay vary widely, but the mean delay noted in the present study is comparable with that observed in Sweden, Finland, Brazil, and others countries (Bilimora et al. 2011; Olsson et al. 2009).

The general level of health education plays a significant role in the management of SCLC, but the main attention should be put on lung cancer prevention, foremost on antismoking policies as 98 % of our patients were smokers. The first symptom(s) of lung cancer are often taken as infection or were connected with age or other coexisting diseases (Turkington et al. 2002). SCLC is a highly progressive disease, and when the symptoms appear, the disease is usually advanced. Probably, in the present study these factors had to do with the lack of the influence of the patient's delay on survival. It has previously been observed that asymptomatic patients in clinical Stage I, who were treated with combined modality therapy, have a better prognosis (Shepherd et al. 2007; Lassen et al. 1995). Our present

data lend support to that notion as this group of patients had indeed a more favorable prognosis than the patients treated only with chemo-radiotherapy or chemotherapy. However, our group of such patients was rather small, amounting to 150 persons.

The median diagnostic delay in our population was 34 days and the median therapy delay was 42 days. These delays that fulfil the BTS (1998) recommendation were due likely to the fact that 50 % of patients are diagnosed and treated in Poland in the Pulmonary Outpatient Departments. However, there were still 50 % of patients in whom diagnostic procedures took over 5 weeks. Also, about 3 % of patients refused therapy and over 10 % were treated symptomatically. Particularly, in these last groups of patients there were some who did not fully cooperate with the diagnosis procedures. Additionally, technical difficulties, additional diseases, errors in interpretation of chest X-rays might extend the time to diagnosis. Nevertheless, in the present study the specialist's delay seems to be too long in comparison with other published data (Olsson et al. 2009). However, SCLC patients were treated as fast as possible, and delay connected with administration of treatment was short (median 6 days).

Multivariate analysis of factors influencing survival revealed that well known predictors of survival such as clinical stage, ECOG PS, and the type of therapy, but not patient's delay influenced survival in SCLC. Paradoxically, we revealed that SCLC patients with prolonged diagnosis and/or longer period to the onset of treatment had a better prognosis. That however was true only for patients in good (0 + 1) or bad (3 + 4) ECOG PS. A probably explanation of this phenomenon is that in patients in bad ECOG PS (50 % of all) diagnostic procedures were applied more promptly than in those severely ill in whom even fast diagnosis could not change a bad prognosis. There also were patients with less advanced disease, but with bad ECOG PS connected with comorbidities. These patients could have better survival than the patients with bad ECOG PS caused by cancer. On the other hand, patients in good ECOG PS might constitute a subgroup of SCLC with a relatively better prognosis, and then even a longer wait time for diagnosis and treatment would not influence survival. Similar observations showing that ECOG PS has an influence on survival and it should factor in treatment decision-making have been reported by Chen et al. (2010).

Recently, Olsson et al. (2009) presented a systematic review of studies on diagnostic and therapeutic delays in lung cancer patients. The authors identified 18 studies examining the association between timeliness and clinical outcomes in lung cancer. The results were mixed. Eight studies showed no association between timeliness and outcome, but data based on lung screening programs showed worse prognosis in patients with delayed diagnosis and therapy. However, four studies, among them Gonzalez-Barcala et al. (2010) and Skaugh et al. (2011), reported worse survival in patients who received timely care (Olsson et al. 2009). It is underlined that patients with urgent referrals have more advanced disease and worse ECOG PS. In fact, there has not yet been any data in the literature on the patient and doctors' delays in SCLC. There are still many problems to be resolved connected with timeliness care. Probably, the development and implementation of new techniques of early lung cancer detection may lead to better outcome in SCLC patients (Rahman et al. 2011; NLST updates 2010).

Acknowledgments The study was supported by Grant 3.6 of the National Institute of Tuberculosis and Lung Diseases in Warsaw, Poland.

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

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Familial Idiopathic Pulmonary Fibrosis 49

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial lung disease of unknown etiology, with an appearance of usual interstitial pneumonia on lung biopsy. To-date, about a 100 families diagnosed with IPF have been described. Familial IPF is defined as histologically confirmed IPF occurring in two or more members of a family. Familial pulmonary fibrosis is hereditary, most probably as a feature which is autosomal dominant with variable penetration. Since 2002, we have been following two families with IPF, referred to in the present article as A and B. The patients in Family A included brother, sister, and sister's daughter. We examined two closest relatives of the patients in family A who are healthy. The patients in Family B included father and his two children. In Family B, we examined six other closest relatives, all of whom proved healthy. In all cases, IPF diagnosis was confirmed histologically. We examined human leukocyte antigen (HLA) alleles in both families, including antigens Class I (locus A, B, and C) and Class II (locus DR). On the basis of the results obtained it is impossible to determine the relation between major histocompatibility complex (MHC) polymorphisms and the incidence of the disease.

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_49,

Keywords
Familial idiopathic pulmonary fibrosis • Human leukocyte antigen • Interstitial lung disease • Histocompatibility complex • Gene polymorphism

1 Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial lung disease of unknown etiology. According to the international consensus statement (American Thoracic Society 2000) IPF is a chronic fibrosing interstitial pneumonia with a histological appearance known as usual interstitial pneumonia. The environmental factors such as exposure to wood or metal dust (Hubbard et al. 1996) play a role in the pathogenesis of IPF. The susceptibility to IPF can be caused by genetic factors. Familial pulmonary fibrosis is hereditary, most probably as a feature which is autosomal dominant with variable penetration. Its incidence is equally often in both sexes. A positive correlation has been observed between IPF and alleles of α1-antitrypsin inhibitor gene and HLA genes, such as B15, B8, B12, DR2, and Dw6 (Hodgson et al. 2005; Geddes et al. 1977). Studies investigating the influence of major histocompatibility complex (MHC) on susceptibility to IPF show contradictory results (Turton et al. 1978). In one such study, evaluating the polymorphism of MHC in a group of 75 patients with IPF, an increased incidence of 3 haplotypes in comparison with healthy patients has been shown (Falfán-Valencia et al. 2005).

To-date, about a 100 families diagnosed with IPF have been described (Hodgson et al. 2005; Wahidi et al. 2002; Marshall et al. 2000). Familial IPF has been defined as histologically confirmed IPF occurring in two or more primary biological members of a family (parents, children, or siblings). The analysis of MHC in the families with IPF increases the chances of connecting certain haplotypes with greater risk of incidence of IPF.

2 Patients and Methods

Two families with IPF, referred to in this article as Family A and Family B, were treated since 2002 at the Clinic of Internal Diseases, Gerontology and Allergology, Wroclaw Medical University in Wroclaw, Poland. Patient A1 was diagnosed with IPF at the age of 60; earlier he suffered from progressive respiratory insufficiency of undetermined etiology. In his medical history he stated that his father, who died at the age of 60 of respiratory failure, had similar symptoms, he never smoked and worked as carpenter. The IPF diagnosis of patient A1 was made based on clinical features, bilateral basal crepitations, restriction in pulmonary function tests, high resolution computed tomographic scans and histological appearances corresponding to that of usual interstitial pneumonia (UIP). After IPF diagnosis, the patient's treatment included systemic corticosteroids and long term oxygen therapy. The patient passed away at the age of 61. His sister (A2) was diagnosed with IPF at the age of 59 and passed away at the age of 71 years. His daughter, Patient A3, was diagnosed with IPF at the age of 41 and passed away at the age of 44 years. In all cases, the diagnosis was confirmed histologically as that of UIP. The other members of the family – two children of Patient A2 (marked as subjects A4 and A5) – are healthy. Figure 49.1 displays a genealogical scheme of Family A.

The patients with IPF in Family B included father (Patient B1) and his two children: daughter (B2) and son (B3). The other son (subject B4) is

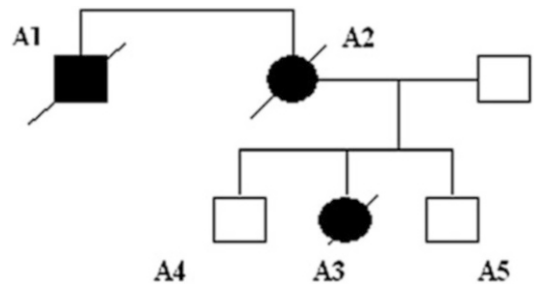

Fig. 49.1 Genealogical scheme of Family A. *Solid symbols* indicate family members suffering from idiopathic pulmonary fibrosis

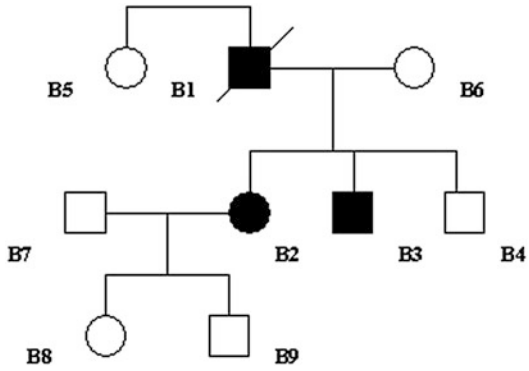

Fig. 49.2 Genealogical scheme of Family B. *Solid symbols* indicate family members suffering from idiopathic pulmonary fibrosis

healthy. Patient B1 passed away at the age of 42; his IPF diagnosis was confirmed histologically when he was 37. Both of his children (B2 – at present aged 26 and B3 aged 24) were diagnosed with IPF at the age of 11 and 9, respectively; both cases confirmed histologically as UIP. Patient B2 was treated with systemic corticosteroids and long term oxygen therapy and was qualified to single-lung transplantation. Patient B3 was also treated with systemic corticosteroids. We also examined the other members of the family: B5 (healthy sister of Patient B1), B6 (wife of Patient B1). Female Patient B2 has two children (B8 and B9) with her healthy husband (B7) (Fig. 49.2).

We examined HLA alleles in both families, including antigens Class I (locus A, B, and C) and Class II (locus DR and DQ). Genomic DNA was extracted from 200 µl of an EDTA whole blood sample using a Isoquick Nucleic Extraction Kit (Invitek GmbH, Berlin, Germany) according to the manufacturer's instructions. In Family A, typing was performed using a microlymphocytotoxicity test for Class I (HLA Ready Plate ABC 120 INNo-Train Diagnostic GmbH, Bad Homburg, Germany), and polymerase chain reaction-sequence specific primer (PCR-SSP) typing for Class II (Biotest DRB SSP Kit, Biotest AG, Dreieich, Germany). The typing of genotype in Family B was conducted 5 years later than in Family A. In Family B the typing was performed using the molecular method PCR-SSP (HLA-Ready Gene ABC Low and HLA-Ready Gene DRDQ Low; INNo-Train Diagnostic GmbH, Bad Homburg, Germany). Primer mixes used to determine the specific HLA Class I (A, B, C) and HLA Class II (DR, DQ) alleles are available in the kit.

PCR amplification was performed using genomic DNA (50 ng/µl), Ready PCR buffer, Axi Taq polymerase (INNO-Train Diagnostic GmbH, Bad Homburg, Germany), 5 U/µl, and sets of primers. The amplification profile consisted of denaturation 96 °C (120 s), next 10 cycles: 96 °C (15 s), 65 °C (60 s), and 20 cycles: 96 °C (15 s), 61 °C (50 s), and 72 °C (30 s). An automated thermal cycler was used for amplification (Biometra T Gradient, Goettingen, Germany). After gel electrophoresis of PCR products in 2 % agarose in 1 × TBE buffer (20 min at 10 V/cm), UV transilluminator (Vilber Lourmat, Marne-la-Vallée Cedex 1, France) was used for visualization of the obtained amplification pattern. The results were interpreted following the instructions of typing sheets from the procedure guide.

3 Results

Tables 49.1 and 49.2 show the results of human leukocyte antigen (HLA) typing in both families. Analysis of the histocompatibility complex (MHC) in the two families, with three persons suffering from idiopathic pulmonary fibrosis (IPF) each, with the same histological appearance typical for usual interstitial pneumonia (UIP), failed to demonstrate any correlation between HLA and susceptibility to IPF in comparison to healthy members of the families.

4 Discussion

Studies on IPF, in most cases on a small number of patients, does not demonstrate any relation between gene polymorphism and susceptibility to IPF (Geddes et al. 1977; Hodgson et al. 2005). Despite the fact that polymorphism of genes coding tumor necrosis factor, interleukin-1 receptor

Table 49.1 HLA typing and haplotype segregation in Family A

Male patient A1 (IPF)	**Female patient A2 (IPF)**	**Female patient A3 (IPF)**
A2;B*56;Cw1;DRB1*04	A2;B56;Cw1;DRB1*04	A3;B35;Cw4;DRB1*01
A2;B18;Cw7;DRB1*11	A3;B35;Cw4;DRB1*01	A28;B44;Cw7;DRB1*11
Male healthy subject A4	**Male healthy subject A5**	
A3;B35;Cw4;DRB1*01	A3;B35;Cw4;DRB1*01	
A28;B44;Cw7;DRB1*11	A28;B44;Cw7;DRB1*11	

Table 49.2 HLA typing and haplotype segregation in Family B

Male patient B1 (IPF)	**Female patient B2 (IPF)**
A*02;B*40;C*03;DRB1*13;DQB1*06	A*25;B*18;C*12;DRB1*04;DQB1*03
?; ?; ?	A*02;B*40;C*03;DRB1*13, DQB1*06
Male patient B3 (IPF)	**Male healthy subject B4**
A*25;B*18;C*12;DRB1*04;DQB1*03	A*68;B*38;C*12;DRB1*11;DQB1*03
A*02;B*40;C*03;DRB1*13;DQB1*06	A*02;B*40;Cw*03;DR1*13;DQB1*06
Female healthy subject B5	**Female healthy subject B6**
A*02;B*40;Cw*03;DRD1*13;DQB1*06	A*25;B*18;C*12;DRD1*04, DQB1*03
A*25;B*38;C*12;DRB1*13;DQB1*06	A*68;B*38;C*12;DRB1*11;DQB1*03
Male healthy subject B7	**Female healthy subject B8**
A*02;B*07;C*07;DR1*14;DQB1*05	A*02;B*07;C*07;DRB1*14;DQB1*05
A*30;B*13;C*06;DRB1*07;DQB1*02	A*25;B*18;C*12;DRB1*04;DQB1*03
Male healthy subject B9	
A*02;B*40;C*03;DRB1*13;DQB1*06	
A*30;B*13;C*06;DRB1*07;DQB1*02	

antagonist, and interleukin-6 was observed in IPF patients, it has been impossible to conclusively connect gene polymorphism with a specific genotype due to a small number of patients (Pantelidis et al. 2001; Whyte et al. 2000). Falfán-Valencia et al. (2005) described, in a group of 75 patients with IPF unrelated with one another, a significantly more frequent incidence of three haplotypes in comparison with healthy subjects: HLA-B*15-DRB1*0101-DRB1*0501, HLA-B*52-DRB1*1402-DQB1*0301 and HLA-B*35-DRB1*0407-DQB1*0302. Bronchoalveolar lavage fluid collected from those patients showed increased apoptosis of epithelial cells in comparison with healthy subjects or patients having different haplotypes. It is known that apoptosis of alveolar epithelial cells plays a key role in the pathogenesis of IPF (Barbas-Filho et al. 2001).

A connection between IPF and the antigens of HLA Class II has also been reported. Libby et al. (1983) described a connection between IPF and the presence of DR2 antigen, but not that of A3 or B7 antigen with which DR2 is usually co-present in haplotype. The existence of such a connection has been confirmed (Xue et al. 2011), demonstrating specifically that DRB1*1501 allele is present more often in sick patients with significantly debilitated gas exchange than in the control healthy subjects. The connection with HLA antigens may, however, be secondary and may actually be with other genes, e.g., MICA, which are present in the HLA region (Aquino-Galvez et al. 2009). Armanios (2012) has described IPF as a disease linked to mutations of telomeres.

The identification of genetic susceptibility to IPF connected with MHC can facilitate earlier diagnosis of IPF and the selection of cases in which the course of the disease would be especially progressive. A rare incidence of IPF, and thus a limited number of patients available for research, constitutes a major problem in achieving progress in the understanding of its pathogenesis. We encountered a similar problem in the present study, as we were able to determine a

potential risk of IPF development, based on HLA, only in Patient B9 (son of Patient B4 diagnosed with IPF) in Family B. Nevertheless, it is the research on the profile of gene expression that may bring us closer to diagnostic and prognostic solutions in IPF (Kass and Kaminski 2011; Yang 2012).

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

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High Resolution Computed Tomography in 2-Year Follow-Up of Stage I Sarcoidosis

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Abstract

There are as yet no markers known to predict the course of sarcoidosis. High resolution computed tomography (HRCT) is a tool that enables to visualize subtle parenchymal opacities in the lungs. Therefore, the aim of this study was to assess the prognostic role of HRCT at Stage I sarcoidosis. Fifty one patients (28 males and 23 females, aged 23–58) were studied. Based on HRCT examinations, two groups were distinguished: HRCT-positive (28 patients with pathologic changes in pulmonary parenchyma – mainly nodular opacities) and HRCT-negative (23 patients without parenchymal opacities). We found no significant differences between HRCT-negative and HRCT-positive groups in the mean values of pulmonary function tests (FEV_1, FVC, FEV_1/FVC, DL_{CO}, and d(A-a)O_2) between the starting and ending measurements of a 2-year long observation (check-up every 3 months). Likewise, there were no differences in the X-ray follow-up between the HRCT-positive and HRCT-negative groups. Nor were there significant differences in the percentage of patients showing stabilization, progression, or improvement between both groups (18 vs. 39 %, 21 vs. 4 %, and 61 vs. 57 %, respectively). We conclude that HRCT examination in stage I sarcoidosis has no significant prognostic role during a 2-year follow-up.

Keywords

High resolution computed tomography • Prognosis • Pulmonary parenchyma • Pulmonary function tests • Sarcoidosis

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_50,

1 Introduction

Sarcoidosis is a multiorgan granulomatous disease of unknown etiology. It predominantly develops within lungs in 70–90 % of patients, but it can affect virtually any organ. A natural course of sarcoidosis, excluding patients with acute sarcoidosis such as, e.g., Löfgren's syndrome, is variable and unpredictable (Judson et al. 2003). Stage I sarcoidosis, consisting of only hilar enlargement on chest X-ray, resolves usually spontaneously in 55–90 % of patients, but progression can occur in the remaining 10–30 % of cases (Judson et al. 2003; Wells et al. 2008; Hunninghake et al. 1999). High resolution computed tomography (HRCT) enables to visualize the parenchymal changes already at Stage I which cannot be visualized in a conventional X-ray (Joseph and Lynch 2003; Grenier et al. 1999). Therefore, a question of whether revealing such subtle changes in lung parenchyma could predict the eventual progression of the disease becomes relevant. Studies regarding the prognostic role of HRCT in sarcoidosis have so far included groups of patients with diffuse changes in lungs seen also in X-ray, i.e., patients with Stage II or III (Masanori et al. 2005). The usefulness of HRTC in predicting the course of disease in untreated patients with Stage I sarcoidosis has not yet been defined. The aim of this study, therefore, was to assess the prognostic role of HRCT in patients with Stage I sarcoidosis.

2 Methods

A retrospective analysis was carried out in a group of 51 non-smoking patients with histopathologically confirmed sarcoidosis (23 females and 28 males), aged 23–58 who were examined and treated at the Department of Lung Diseases and Tuberculosis, Silesian University of Medicine in Zabrze, Poland. In most cases sarcoid radiological changes (bilateral hilar enlargement only) were discovered during a routine radiological chest examination and the patients did not report any serious complaints. No symptoms or signs of acute or extrapulmonary sarcoidosis were manifest. The disease duration did not exceed 2 years.

All patients had chest X-ray taken using an Mercury 165 machine with a 50HF Genius generator (Villa Sistemi Medicali, Buccinasco, Italy) and stage I of radiological changes, i.e., bilateral hilar lymphadenpathy was confirmed. The presence of diffuse pulmonary infiltrates was positively excluded. The patients had then HRCT taken with an Somatom AR apparatus (Siemens, Forchheim, Germany) equipped with an algorithm for high resolution using a 512×512 computational matrix. HRTC scans were taken from the top of the lungs to the diaphragm in the patient lying on the back. The thickness of scans was 2 mm and intervals between the scans were 10 mm. The applied window width was between 1,200 and 1,600 HU, while the height was from -800 to -600 HU, and time duration between scans was of up to 3 s. Preliminary evaluation was based on scans showing both lungs, while detailed analysis was carried out on chosen scans limiting the examined scope to 25 cm. Examination results were assessed and described independently by two radiologists. The presence of at least 6 nodules over 3–4 mm in diameter was classified as HRCT positive changes. The patients in whom no parenchymal abnormalities were observed were classified into the HRCT negative group.

The patients underwent spirometric examinations including FVC (forced vital capacity), FEV_1 (forced expiratory volume in 1 s), and FEV_1/FVC using a Transferscreen II setup (Jaeger, Hoechberg, Germany) according to the American Thoracic Society (1995a) guidelines. Lung diffusing capacity for carbon monoxide (DL_{CO}) was measured using a single-breath method with a Profiler apparatus (MedGraphics, St. Paul, MN) according to the American Thoracic Society (1995b) guidelines. The results were presented as the percentage of predicted values (Quanjer 1993). Blood gases analysis was performed with an AVL Compact 1 apparatus (AVL, Graz, Austria). The alveolar-arterial difference ($d(A\text{-}a)O_2$) was calculated according to Stein et al.'s formula (1995).

As a result of the conducted tests, all patients were qualified as not requiring treatment and the follow-up examinations were scheduled. These examinations included medical history, physical examination, and chest X-ray to be performed 3 months after the diagnosis and then in case of regression or stabilization every 6 ± 1 months. Spirometry was performed every 12 ± 2 weeks and diffusing capacity was checked every 6 ± 1 months. Improvement, stabilization, or deterioration of functional tests or radiological pictures was used to define the course of the disease. An increase in FVC by >10 % and/or increase in DL_{CO} by >15 % in relation to the initial values was taken as improvement of pulmonary function tests and a decrease of FVC by ≥10 % and/or of DL_{CO} by ≥15 % as deterioration. The changes of FVC and DL_{CO} which did not fulfil the criteria of improvement or deterioration were classified as stabilization (Hunninghake et al. 1999). Normalization or reduction of hilar lymph nodes by at least 50 % in diameter in comparison with the initial chest X-ray was taken as radiological remission, no changes as stabilization, while the appearance of new parenchymal changes regardless of the size of hilar lymph nodes as progression.

In case of a significant radiological progression or deterioration in pulmonary function tests, the observation was discontinued and treatment with glucocorticosteroids was initiated. This ended the follow-up; the results of the last examination were included in the final assessment. The patients were monitored, on average for, 2.0 ± 0.3 years. The shortest observation period was 6 months (2 patients), while the longest was 38 months (1 patient). Data from 248 check-up visits were analyzed.

Categorical variables were compared with a Chi^2 test. Quantitative changes, after defining normality of distribution by a Szapiro-Wilk test, were compared with a Mann–Whitney *U* test. Results were presented as mean values ±1.96 (SE). $P < 0.05$ was assumed as statistically significant. A commercial Statistica 8.0PL packet was used for all analyses.

3 Results

On the basis of HRCT, we classified 51 patients with Stage I sarcoidosis into two groups: HRTC positive – 28 patients with visible parenchymal opacities and HRCT negative – 23 patients without opacities. In the HRCT positive patients, the most frequently observed abnormality, apart from hilar enlargement, were nodular changes in lung parenchyma seen in 22 (78.6 %) patients. In 13 patients (46.4 %) of this group, nodular changes were additionally located to the bronchovascular bundles. In another 5 patients (17.6 %) of this group, nodular parenchymal changes coexisted with subpleural nodules. Linear shadows were seen in the remaining 6 (21.4 %) patients of the HRTC positive group.

At the beginning of the observation period there were no spirometric disturbances in both HRCT positive and HRTC negative groups. Only had two HRCT positive patients a slightly lowered forced vital capacity (FVC). The diffusing capacity was slightly below the normal range in 12 HRCT positive patients (42.9 %) and in 10 HRCT negative patients (43.5 %). Two patients, one from each group, had a higher alveolar-capillary difference at rest. The mean values of the functional pulmonary tests were similar in both HRCT positive and HRCT negative. Likewise, at the end of the follow-up the mean values of the functional pulmonary test did not differ appreciably from those recorded at the start-up between the corresponding groups of patients (Table 50.1).

During a 2-year follow-up, no statistically significant differences were noted in the frequency of regression, stabilization, and radiological progression between the HRCT positive and HRCT negative patient groups. Nevertheless, in the HRCT positive group a trend was observed for radiological progression (6 patients – 21.4 %), while radiological progression was observed just in one patient in the HRCT negative group (4.3 %); the difference failed to reach the level of statistical significance (Table 50.2).

Table 50.1 Functional pulmonary tests in patients with Stage I sarcoidosis with parenchymal opacities revealed by the high resolution tomography examination (HRCT-positive group) and without parenchymal changes (HRCT-negative group) during a 2-year follow-up

	HRCT positive patients (n = 28)		HRCT negative patients (n = 23)	
	Before	After 2-year follow-up	Before	After 2-year follow-up
FVC (%predicted)	103.6 ± 13.3	102.0 ± 14.0	105.0 ± 9.6	105.1 ± 9.5
FEV_1 (%predicted)	97.6 ± 13.2	95.0 ± 14.4	98.8 ± 10.8	98.0 ± 10.3
FEV_1%FVC (%)	79.0 ± 4.2	78.2 ± 3.7	82.1 ± 3.3	82.0 ± 4.0
DL_{CO} (%predicted)	80.0 ± 15.9	78.0 ± 16.4	82.8 ± 12.4	84.0 ± 10.4
$D(A\text{-}a)_2$ (mmHg)	19.2 ± 10.4	18.0 ± 10.1	16.9 ± 9.2	15.0 ± 8.8

Data are mean values ±SE; differences between the two groups were insignificant ($p > 0.05$)

Table 50.2 Clinical course of Stage I sarcoidosis with parenchymal opacities revealed in high resolution tomography examination (HRCT-positive group) and without parenchymal opacities (HRCT-negative group) during a 2-year follow-up

	HRCT positive patients ($n = 28$) (%)	HRCT negative patients ($n = 23$) (%)
Progression	6 (21.4)	1 (4.3)
Stabilization	5 (17.9)	9 (39.1)
Remission	17 (60.7)	13 (56.6)

Differences between the two groups were insignificant ($p > 0.05$)

Table 50.3 Changes in lung function (FVC and DL_{CO}) in patients with the Stage I sarcoidosis with parenchymal opacities revealed by the high resolution tomography examination (HRCT-positive group) and without parenchymal opacities (HRCT-negative group) during a 2-year follow-up

	HRCT positive patients (n = 28) (%)	HRCT negative patents (n = 23) (%)
Deterioration	2 (7.1)	2 (8.7)
Stabilization	22 (78.6)	17 (73.9)
Improvement	4 (14.3)	4 (17.4)

FVC forced vital capacity, DL_{CO} lung diffusing capacity for carbon monoxide. Differences between the two groups were insignificant ($p > 0.05$)

Due to the radiological progression, the observation was discontinued after 6 months in case of three patients, after 1.5 years in one patient, and after 2 years in another three patients.

As far as the functional pulmonary tests are concerned, the percentage of patients with improvement, deterioration, and stabilization was similar, so that no statistically significant differences were noted. Three patients (two in the HRCT negative and one in the HRCT positive group) demonstrated some deterioration in functional tests (decreases in DL_{CO} and FVC). These changes occurred in one patient after a year and in the other two patients after 2 years of the follow-up (Table 50.3).

4 Discussion

Staging of sarcoidosis, based on chest X-rays, has so far seemed to serve as a simple and useful prognostic means (Wells et al. 2008; Chappell et al. 2000; Hunninghake et al. 1999). In the present study during about 2 years of the follow-up period, 30 patients out of the 51 with the Stage I sarcoidosis had a complete remission seen on a conventional chest X-rays. The role of chest X-rays in predicting the course of sarcoidosis in case of small nodular changes in lung parenchyma present in Stage I of the disease is questionable. Therefore, in this study we

compared the prognostic power of HRCT head-to-head with chest X-rays, and in combination with lung function tests, in predicting the course of Stage I sarcoidosis. We failed to confirm the advantage of HRCT as a predictive means of the disease course.

Studies suggest that a HRCT image is so distinctive that it facilitates a conclusive diagnosis of sarcoidosis (Joseph and Lynch 2003; Brauner et al. 1992; Murdoch and Muller 1992). HRCT may increase the likelihood of correct diagnosis from 30 to 64–72 % in comparison with an assessment based on a conventional lung X-ray. Nodular changes in HRCT can correspond to either stable reversible changes or progressive changes (Grenier et al. 1999; Hunninghake et al. 1999; Remy-Jardin et al. 1994). According to Brauner et al. (1992), nodular changes follow the remission achieved with glucocorticosteroid therapy. Ground glass opacities, thickenings of septal lines, and infiltrations have a better prognosis, as they can regress either spontaneously or as a result of a therapy. Different results were obtained by Masanori et al. (2005), who found in a 7-year long study of 40 sarcoidosis patients that the initial ground glass opacities and consolidations have poor prognosis and evolve into honey-combing. That study, however, did not encompass patients with the Stage I sarcoidosis. Studies on the prognostic value of HRCT have mainly described its usefulness in the therapeutic monitoring of patients with more advanced Stages II-IV of sarcoidosis or in patients treated with glucocorticosteroids (Joseph and Lynch 2003; Murdoch and Muller 1992). Nodular changes revealed in HRCT have been thought to point to the possibility of good prognosis, while irregular shadows might augur progression. Murdoch and Muller (1992) have found HRCT helpful in predicting prognosis, but argue that due to the cost and patient's exposure to radiation the method cannot be routinely used to monitor the effectiveness of therapy. According to the present guidelines, HRCT is not obligatorily recommended for sarcoidosis monitoring (Wells et al. 2008; Hunninghake et al. 1999).

Likewise, lung function tests, carried out at the onset of observation, have no predictive power concerning the course of sarcoidosis or the response to therapy (Baughman et al. 2010; American Thoracic Society 1995a, b). Impairment of lung function, in general, poorly correlates with radiological lung changes (Bergin et al. 1989). In the present study, pulmonary function tended to be slightly worse in patients from the HRCT positive group (Table 50.1), which was of no meaning concerning the prediction of the course of disease (Table 50.3). Similar findings have been reported by others. Abnormalities in lung function, most frequently a reduction in diffusing capacity, occur only in about 20 % of patients with Stage I (Hunninghake et al. 1999; Winterbauer and Hutchinson 1980). A study of Remy-Jardin et al. (1994), in which 95 patients with all categories of changes in HRCT, i.e., septal and non-septal linear shadows, nodules, ground glass opacity, honey-combing, and architectural distortion were investigated, demonstrates the lack of a significant correlation between the scope of nodular changes and functional pulmonary impairment. The issue is, however, still debatable as another study reported a correlation between nodularity and honeycombing observed in HRCT and lung function abnormalities (Ziora et al. 2005).

It is worth mentioning that Brauner et al. (1992) described abnormalities in diffusing capacity in patients even without parenchymal changes seen in HRCT. Such abnormalities may result from subtle nodular changes around bronchioles and pulmonary alveoli, which were missed in HRCT scans taken every 1 cm (Joseph and Lynch 2003; Remy-Jardin et al. 1994). Functional impairment of the respiratory system is often determined not so much by the number of nodules as by their location. Hence, granulomas located around bronchioles can cause disturbance in ventilation and perfusion, resulting in a decrease in the partial pressure of oxygen and an increase in the alveolar-arterial gradient, particularly during effort breathing. Nodules located around vessels can cause perfusion disorders.

We conclude that revealing small opacities in HRCT in patients with Stage I sarcoidosis, not seen in conventional chest radiographs, has no appreciable prognostic value regarding the prediction of the future course of the disease.

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

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Altruistic Aptitude: Age-Dependent Influence of Temperament and Emotional Intelligence

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Mieczyslaw Pokorski, Aneta Faron-Lasyk, and Lukasz Borecki

Abstract

It is unclear why some people behave altruistically and others do not. This study seeks to determine what psychological features could help predict altruistic behavior. We addressed the issue by examining distinct dimensions of temperament and emotional intelligence and their associations with the level of proaltruistic aptitude in two distant age-groups, young (20–29 years) and senior (60–79 years) persons. The study was one of a self-reported psychometric survey. The major findings were that emotional intelligence, rather than temperament, is strongly associated with the expression of altruistic behavior in both young and senior subjects, despite a general decrease in the characteristics of emotional intelligence in advanced age. We also failed to substantiate the presence of an appreciable difference in the level of declared altruism between the senior and young subjects. High emotional intelligence, often underling social engagement and bonding, seems thus a good predictor of altruistic aptitude to be displayed by a person. The independence of this association of age-changes in emotional agility is suggestive of causal relationship. The study is relevant for an understanding of the enigmatic origins of important social behaviors like altruism.

Keywords

Age • Altruism • Emotional intelligence • Social behavior • Temperament

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_51,

1 Introduction

To carry aid or to come to one's rescue in a gratuitous way is a frequent and rising occurrence in the contemporary world. This study seeks to determine what psychological features would be advantageous to make an individual express altruistic aptitude. A standard definition of altruism was adopted for the study, coined by Augustine Comte in the nineteenth century, as the way of carrying selfless help to other people not expecting any reward or benefits in return; the act of helping being of commendable moral excellence in itself. The psychological background of altruism is not straightforward and it is an area of seldom studies, as the expression of altruism is entangled with prosocial or extravertive poses, altruism is subject to social approval, and it may have a covert driving component of complacency which puts a blur on the gratuitous basis of it. Little is known about psychobiological underpinnings of altruism which can interfere with its expression.

Temperament is considered an emotional sphere of personality which sets the ability to respond to emotional arousal and it is viewed as a constitutive psychobiological element (Allport 1937, 1966). Temperament-emotion linkage implies that temperament has to do with the style of one's conduct expressed in his behavioral changes, which also may modify the influence on an individual of external events (Rothbart et al. 2000). These characteristics of temperament are at the center of a recent regulative theory of temperament of Strelau (1996, 1998) which entails biologically determined temporal and energetic features underlying human behavior. According to the theory, temperamental features counterbalance deflections from the individual's optimum in psychological responsiveness by behaviorally driven adjustments, which are crucial for the effective processing of emotional stimulation (Strelau 2008).

So far, little attention has been paid to the aspects of temperament responsible for individual differences in the expression of pro-altruistic aptitude. Temperament is instrumental in regulating one's contacts with the surrounding world and in that sense it interferes with, and connects to, emotional intelligence. Solovey and Mayer (1990) conceptualize emotional intelligence as the ability to understand one's own and others' feelings and to use this knowledge to guide one's actions in a personally and socially rational way. An association between temperament and emotional intelligence seems multi-pronged. If task-oriented and pro-social actions drive an individual, then the intensity of one's engagement would have to do with the features of temperament related to the ability to acquire and process the stimulatory drive. Likewise, perception and reliving of emotions are underlain by one's temperament. There is also a coexistence of the emotional affects with the sensibility underlain by temperamental traits. An individual should be able to control his emotional stance at the optimal level to uphold activity commensurable with the accompanying emotions. It is unclear, however, which traits of temperament would dominantly associate with emotional intelligence (Matczak 2007).

In the present study we set out to determine the associations of distinct dimensions of temperament and emotional intelligence with the prosocial or altruistic behavior. Additionally, we examined the effect of age on these associations. Our working hypotheses were that altruistic aptitude increases with increasing level of social intelligence and the senior persons would articulate a more altruistic aptitude compared with the young ones. We addressed the issues by carrying out psychometric analyses in two distant age-groups, young and senior persons.

2 Methods

The study was approved by the Institutional Review Board for Research of the Institute of Psychology of Opole University, Poland and was performed in accord with the Declaration of Helsinki for Human Research. The psychometric method of the study consisted of a self-reported survey. All participants were informed about the general survey procedures and the anonymity and voluntarism of responses, but

not about the detailed hypotheses set for the study. They gave informed consent for being surveyed.

A total of 120 adult subjects were enrolled into the study. They were asked to complete a demographic survey and they were then broken down into two age-groups: young (59 subjects, F/M – 33/26, age range 20–29 years, mean 24.0 ± 2.5 SD years), all university students or graduates, and senior (61 subjects, F/M – 50/11, age range 60–79 years, mean 67.0 ± 6.1years) all listeners of the University of the Third Age in the city of Opole, Poland. The participants were in good general health, with no overt diseases or health problems, particularly in the psychology-related domains, and free of pharmacological treatments. Both groups were fairly homogenous regarding the education which was college or university, the place of abode, which was a medium size city, and the socioeconomic status, which was a middle class.

There were three main variables assessed in the study: temperament and its constituents, emotional intelligence and its constituents, and altruism. The altruism was set as the measure differentiating the results of the other variables in the two age-groups. The power of each variable was assessed from a corresponding questionnaire. Temperament was assessed with the Formal Characteristics of Behavior-Temperament Inventory (FCB-TI) of Strelau and Zawadzki (1993). This inventory was constructed on the basis of the Regulative Theory of Temperament (RTT), created by Strelau (1996, 1998), which introduces six biologically determined dimensions of temperament underlying human behavior: briskness, perseveration, sensory sensitivity, emotional reactivity, endurance, and activity. Each dimension has its own subscale in the inventory, consisting of 20 items, giving a total of 120 items with the yes or no response pattern. The inventory has been extensively validated and it is suitable for persons between 15 and 80 years of age (Strelau and Zawadzki 1995).

Emotional intelligence was assessed with the Popular Inventory of Emotional Intelligence of Jaworska and Matczak (2005) which was constructed on the base Solovey and Mayer's (1990) concept of emotion intelligence. The inventory consist of four subscales defining the following dimensions of emotional intelligence: 1/ acceptance, expression, and use of one's emotions in responding (15 items), 2/ empathy – the ability to understand and monitor others' feelings and emotion (18 items), 3/ control over the expression and driving of one's own emotions (11 items), and 4/ understanding and discriminating among one's own live-through emotions (10 items). The inventory consists of 94 items in total, as there are 40 additional items unrelated to any of the categories above outlined. The subject's responses are measured on a 5-degree scale. The inventory has a high internal consistency, verified for both young and senior persons of both genders, with α-Cronbach coefficient of 0.90 for the total score and between 0.70 and 0.80 for particular subscales where adult subjects are placed in the higher end of the range.

The level of altruism was assessed with the Questionnaire of Altruism and Nonaltruism, A-N questionnaire, by Sliwak (2005). Altruistic behavior is understood in this questionnaire as one's mindful and voluntary action performed for the benefit of others, without any expected reciprocal reward or gratification other than one's sense of doing good. The A-N questionnaire consists of two sets of short essays. There are nine essays in the first set, each having six ready descriptions expressing a different level of altruistic aptitude of the character's action. The surveyed subject should be asked to choose the most suiting to him description. In the second part, there are eight essays in which the resolution of the character's action dilemma had already been done by him. The surveyed subject should now be asked to take stand on that resolution. The answers are given on a 7-degree Likert scale. The total score ranges from 17 to 110 points; the higher the score the higher is the level of altruism. The questionnaire has a verified high reliability and was contrived in the way to minimize the influence of social approval usually accompanying the measures of altruism in self-reporting.

Subjects were surveyed in groups consisting of several persons. Each subject obtained a set of

questionnaires in the order outlined above. It took a person about 40 min to complete all questionnaires. Data were presented as means ±SD of raw scores. Statistical elaboration consisted of an unpaired *t*-test for intergroup comparisons of the corresponding variables and of Pearson's *r* correlations for the assessment of associations between individual variables. The level of statistical significance was set at $P < 0.05$.

3 Results

3.1 Descriptive Comparison of Age-Groups

The age-groups differed regarding the intensity of temperamental features. The differences are depicted in Table 51.1. Young subjects had a significantly better score in briskness and endurance compared with the seniors. The latter group, on the other side, had an edge in emotional reactivity ($P < 0.01$ for all).

Likewise, there were differences in the constituents of emotional intelligence. The abilities to control and understand emotions were significantly greater in the young compared with those in the older subjects ($P < 0.005$). The young ones also had a greater total score of emotional intelligence ($P < 0.05$). The level of altruism, however, did not differ between the two age-groups (Table 51.1).

3.2 Psychometric Correlations

Analysis of behavior-related characteristics of temperament revealed that briskness, sensory sensitivity, and endurance correlated positively, and perseveration and emotional reactivity correlated negatively with emotional intelligence in the young subjects. In the senior subjects, emotional reactivity also correlated negatively and sensory sensitivity correlated positively with emotional intelligence (Table 51.2).

With regard to the cross-correlations between the individual features of temperament and those of emotional intelligence, we found that the negative relations of emotional reactivity to the emotion control and understanding in both young ($r = -0.618$ and $r = -0.532$, respectively, $P < 0.01$) and senior ($r = 0.443$ and $r = 0.432$, respectively, $P < 0.01$) persons had the most decisive influence on the total emotional intelligence's associations presented in Table 51.2,

Table 51.1 Characteristics of altruism, temperament, and emotional intelligences in the 20–29 years and 60–79 years age-groups

	Young ($n = 59$)	Senior ($n = 61$)	P<
Altruism			
	72.7 ± 9.0	72.8 ± 10.2	NS
Temperament			
Briskness	**15.2 ± 3.6**	13.4 ± 3.7	0.01
Perseveration	13.5 ± 4.1	12.6 ± 3.4	NS
Activity	9.8 ± 4.9	8.4 ± 3.9	NS
Emotional reactivity	10.0 ± 4.8	**12.6 ± 3.8**	0.01
Sensory sensitivity	15.0 ± 3.5	14.1 ± 3.2	NS
Endurance	**10.0 ± 5.3**	7.2 ± 4.0	0.01
Emotional intelligence			
Acceptance	53.1 ± 8.3	51.1 ± 6.9	NS
Empathy	69.3 ± 7.9	71.3 ± 8.9	NS
Emotion control	**35.1 ± 7.6**	31.1 ± 6.5	0.005
Emotion understanding	**33.1 ± 7.2**	29.1 ± 6.7	0.005
Total	**338.9 ± 37.3**	328.1 ± 33.4	0.05

Data are means ±SD of raw scores. Bold indicates data that statistically differ between the two groups

Table 51.2 Associations between temperamental characteristics and emotional intelligence

	Emotional intelligence			
	Young ($n = 59$)		Senior ($n = 61$)	
Temperament	*r*	P<	*r*	P<
Briskness	0.428	0.01	0.025	NS
Perseveration	−0.291	0.05	0.014	NS
Activity	0.133	NS	0.102	NS
Emotional reactivity	−0.525	0.01	−0.309	0.05
Sensory sensitivity	0.258	0.05	0.275	0.05
Endurance	0.339	0.01	0.027	NS

Pearson's *r* correlation coefficient, *NS* nonsignificant

Table 51.3 Associations between dimensions of temperament and of emotional intelligence and altruism in young and senior persons

	Altruism			
	Young ($n = 59$)		Senior ($n = 61$)	
Temperament	*r*	P<	*r*	P<
Briskness	0.101	NS	0.022	NS
Perseveration	−0.209	NS	0.015	NS
Activity	−0.003	NS	−0.073	NS
Emotional reactivity	−0.356	0.01	0.073	NS
Sensory sensitivity	−0.057	NS	0.284	0.05
Endurance	0.320	0.05	−0.012	NS
Emotional intelligence				
Acceptance	0.264	0.05	0.318	0.05
Empathy	0.383	0.01	0.517	0.001
Emotion control	0.446	0.001	0.254	0.05
Emotion understanding	0.457	0.001	0.342	0.01
Total	0.554	0.001	0.596	0.001

Pearson's *r* correlation coefficient, *NS* nonsignificant

with the remaining constituents of emotional intelligence having a variable influence.

A correlation analysis also was performed to assess the influence of temperamental and emotional intelligence features on the declared level of altruism in the subjects of both age-groups. The results of this elaboration are displayed in Table 51.3. Temperament showed a rather meager influence on altruistic aptitude, with emotional reactivity correlating negatively and endurance correlating positively in the young and sensory sensitivity correlating positively with altruism in the senior subjects. By contrast, all constituents of emotional intelligence distinctly correlated with the level of altruism in all studied subjects, in the way that the higher the emotional intelligence the higher was the declaration of altruistic aptitude. The strength of these correlations was statistically greater in the young subjects compared with the old ones. An appreciable enhancing effect on altruism exerted by the level of emotional intelligence, taken as a total of its constituent dimensions, is visualized in Fig. 51.1. The effect was of similar strength in both young and senior subjects, as there was no statistical difference between the 0.13 and 0.18 figures describing the linear slope lines of altruism on emotional intelligence in both age-groups.

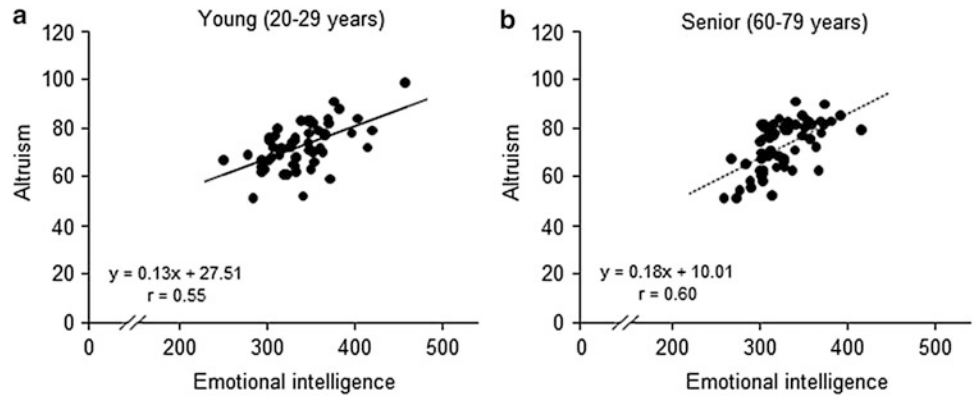

Fig. 51.1 Relationship between declared altruistic aptitude and the level of emotional intelligence in young (*Panel a*) and senior (*Panel b*) persons. *Lines* are linear regression slopes. No statistical difference in the relationship between the two age-groups was found

4 Discussion

The present study was undertaken from the perspective of connections between temperament and emotional intelligence, the constructs determined mostly genetically, albeit influenced by the socialization process and age, with altruism. The study demonstrates that emotional intelligence and its constituents, rather than temperament, have a proaltruistic effect in both young and senior subjects, despite a general decrease in the characteristics of emotional intelligence in advanced age. The results also failed to substantiate the presence of an appreciable difference in the level of declared altruism between the senior and young subjects. This finding runs against our working hypothesis that the self-reported level of altruistic aptitude would be enhanced in senior subjects. There are, often popularly expressed, reasons to believe it might be so. Older persons are considered more empathic, more understanding and considered for the feelings of other, and more engaged in interpersonal and social activities due to staying away or shunning the bustling and competitive life style characteristic of their working days, all of which may foster altruistic aptitude. This kind of aptitude may also have to do with the richness and variability of human experience that accumulates over years. The presently surveyed results counter the reasoning above outlined.

Not only was altruism not enhanced in senior subjects, but also they appreciably lagged behind the young ones in the ability to control and understand emotions. These are cognitive aspects of emotional intelligence and the ability to process information decreases in older age, which translates into a longer time lag to percept a stimulus and respond in a sensorimotor way (Salthouse 1996). A lower level of emotional intelligence in older subjects is also explainable by their tendency to maximize positive emotions in social contacts, which is linked to brushing aside negative emotions that are indispensable for emotion control and understanding (Labouvie-Vief et al. 1989). There are, however, studies that rather point to older subjects as having higher emotional control (Szczygiel and Jasielska 2008; Gross et al. 1997). The divergent results may be explained by methodological differences. For instance, Szczygiel and Jasielska (2008) have compared emotional intelligence in subjects aged, on average, 19 and 52 years; the latter group is hardly comparable with the age of our senior subjects.

Although the present results clearly point to a significant edge in emotional intelligence displayed by young subjects, caution should be exercised in the interpretation. It may be that not so much

emotional intelligence, but its use might be impaired in senior subjects due to the above mentioned difficulties in cognition in older age. On the other side, a high level of emotional intelligence in young persons may have some less genuine attributes like the desire for autopresentation, which cannot be adequately controlled for. There also is a current tendency of putting emphasis on the development of social competence to enhance professional effectiveness and achievements in young people entering the work market. An easy access to instructions and workshops on emotional intelligence may be spuriously reflected in self-reported higher scores. The exact determinants of the differences in emotional intelligence between the young and old subjects should be explored in other study designs.

There were also changes in the dimensions of temperament between the young and senior age groups of this study. Briskness, the ability to react swiftly and changeably, and endurance, the ability to react adequately to the duration and sustenance of the stimulus were significantly more expressed in the young than senior subjects. On the other side, reactivity to emotional stimuli was higher in senior subjects. Since emotional reactivity correlates negatively with emotion control and understanding, senior subjects are at a clear disadvantage in this respect. These differences in temperament, albeit weaker expressed than those in emotional intelligence, are in rapport with the latter and they point to changes in the style of behavior or the way of acting in response to emotional stimulation with advancing age. The style would be more straightforward in young subjects, characteristic of low emotional reactors, whose efficiency of acting drops at a low level of stimulation and whose preference is for a behavioral adaptation reflecting a high emotional content. By contrast, a higher emotional reactivity in senior subjects would come down to a greater sensibility, but lower resilience, features characteristic of auxiliary style of acting according to the Strelau theory (Strelau 2008). These results lend support to the notion put forward in other studies that genetic and environmental factors collaborate in setting the temperamental phenotype. The mutual relationship of these factors is highly variable, depends on individual features and environmental circumstances, and its assessment is difficult due to the lack of longitudinal studies (Oniszczenko 2007). Nevertheless, a review of various studies sets the ratio of genetic to environmental factors in personality changes at 2:3 (Loehlin 1992). In general, it is assumed that the proportion of genetic traits is less than that of the environmental ones, but the latter assume an upper hand with advancing age due to the accumulated specific lifetime environmental experiences, with a possible exception of intelligence, evaluated as the intelligence quotient, for which genetic traits plays a greater role with age (Bergeman et al. 2001).

Greater emotional intelligence is useful in the controlled processing of negative emotions, and thus it would possibly enhance motivational and altruistic activities. Acting for the welfare of others, requiring often sacrifice or emotional endurance in the short term, may be emotionally satisfying later on, yielding a sense of realization of a greater goal. Emotional intelligence appraises the cognitive processes that underlie our personal characteristics and drive behaviors. Temperament is instrumental in regulating one's contacts with the surrounding world and in that sense it interferes with, and connects to, emotional intelligence (Rothbart et al. 2000). The correlations we found in the present study were modest, but they suggest that temperamental characteristics show associations with emotional intelligence rather than altruism and that these associations are expressed more in the young than senior subjects. Young persons have a greater ability to process feelings and to respond to extraneous emotional stimulation in relation to the temperamental status. The results demonstrate that emotional intelligence drives altruistic behavior and does that to a similar degree in both age-groups, even thought the level of emotional intelligence is lower in senior age. These results corroborate the notion of a close relation of emotional expressiveness to the intensity of social interactions, empathy, and social competence (Matczak 2007; Strayer and Roberts 2004), all of which underscores the desire to work for the benefit of others. Meager connections we found between the qualities of temperament and altruism also speak in favor of

prosocializing activities as a motivational driving force for the expression of altruistic aptitude.

Why do some people act altruistically and others do not show such an aptitude is unclear. From the pragmatic standpoint it seems desirable, in some situations, to be able to sort out the altruistic lot. Psychological altruism, as a separate brain entity, perceived as a prosocial behavior directed at comforting other people's needs in a selfless way, has recently gained support from neurophysiological research. Moll and Grafman (2006) have shown that gratuitous charitable deeds activate selectively the subgenual cortex, the area distant from the mesolimbic reward pathway activated by reward expectation. In another study, the posterior superior temporal cortex activity increased in proportion to a subject's self-reported level of altruism, pointing to the neurophysiological disparity between the ideation and execution in reality of altruistic action (Tankersley et al. 2007). Brain activation stemming merely from one's outlook on the world may be instrumental in the expression of social behaviors like altruism. Likewise, studies on the neurophysiological correlates of emotional intelligence are even scanter. In a study based on identifying emotions in pictures, the level of emotional intelligence was linked to specific changes in gamma/alpha band synchronization/desynchronization EEG pattern (Jaušovec and Jaušovec 2005). High emotional intelligence evokes more gamma band synchronization and less alpha band desynchronization, which is in line with using more resource full strategies to problem-solving by individuals of higher emotional intelligence. Neurophysiological examinations are, however, hardly practical tools to unravel altruistic aptitude of a person.

Although the current study does not resolve the enigmatic origins of important social behaviors like altruism, we believe we have conclusively shown a positive association between the level of emotional intelligence and altruism. High emotional intelligence seems thus a good predictor of altruistic aptitude to be displayed by a person. Moreover, the relationship holds true independently of age, even if emotional intelligence declines in senior age due to a general decline in cognitive agility, which, in our view, gives credence to the specificity of emotional intelligence-altruism connectivity. Our results also play down the role of temperamental traits in setting altruistic behavior. It seems emotional intelligence represents an essential element underlying brain altruistic architecture.

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

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Effect of Socio-Economic Status on Quality of Life in People Affected with Respiratory Allergy

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Abstract

In the present study we investigated the impact of respiratory allergy on quality of life in young people, and examined whether socio-economic status modifies the above dependence. The study was conducted in 458 female and 363 male university students, aged 18–25. Information on socio-economic status (SES) was collected using a questionnaire. The occurrence of allergy was determined on the basis of answers to the questions whether the allergy and specific allergens were medically diagnosed. Quality of life (QoL) was based on the Polish version of the SF-36 test. Respiratory allergy or respiratory and food allergy were declared by 19.2 % of women and 19.0 % of men. The prevalence of allergy was higher in students with high SES. The students suffering from allergy obtained lower scores in all domains of QoL, but the differences were statistically insignificant. However, the overall test result in allergic students was significantly lower than that in non-allergic students. Differences QoL were significantly associated with socio-economic variables. In persons with low SES, the differences in QoL between those suffering from allergy and those who did not have allergy were larger than in persons with high SES. The results indicate that the course of allergic diseases is highly dependent on socio-economic status. The prevalence of allergy among students of low status is lower than among those of high status. However, allergy to a greater extent impairs the quality of life of students with low than high SES.

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DOI 10.1007/978-94-007-6627-3_52,

Keywords
Respiratory allergy • Quality of life • Socio-economic status • University students

1 Introduction

The number of people suffering from allergic problems increases each year (Asher et al. 2006). It is estimated that currently the number of people who suffer from asthma or allergic diseases is more than 700 million worldwide. These diseases are not the cause of high mortality, but significantly affect the quality of life (Majani et al. 2001; Sicherer et al. 2001; Leynaert et al. 2000). Symptoms associated with allergy make it difficult to function in daily life and lower the quality of life.

Research into the causes and consequences of allergic diseases have become the subject of many scientific papers in recent years. This is a consequence of a rapid increase in frequency of allergies in both children and adults. Genetic predispositions appear essential for the development of allergies. The incidence of allergies among children, whose parents do not suffer from these diseases is 5–15 %, when allergy occurs in one of the parents – 20–40 %, and when both of parents suffer from allergy – it is at least 60 % (Pawlinska-Chmara et al. 2008; Steinke et al. 2008). Allergy is a disorder conditioned by a number of genes and its incidence is also influenced by environmental factors. Most of them are linked to the progress of civilization: air and water pollution, addition of preservatives, artificial colorings and other additives, exposure to tobacco smoke, and a massive use of antibiotics. A major role is also attributed to factors related to lifestyle, such as low physical activity, frequent stay in closed rooms with sealed windows or air conditioning, or a diet based on highly processed foods (Samolinski et al. 2012; Strachan 2000; ISAAC 1998).

Allergy is often referred to as a disease of prosperity, due to the fact that the increase in its incidence has been observed mainly in countries with high levels of life. In addition, within a single country, allergy is observed more often in persons of high socio-economic status (SES), than in low-status groups (Gold 2006; Lannerö et al. 2002; Lewis et al. 2001; Strachan 2000; Heinrich et al. 1998; Ernst 1996). Allergy is one of the few diseases associated with high SES, as such persons, in general, have better health than those of SES (Lynch and Kaplan 2000). That is connected primarily with more health care, arising from both greater knowledge of pro-health behaviors and financial capacity, and thus from better access to medical care, early detection of diseases, and their treatment. Differences depending on the status can also be seen in the diet, physical activity, housing conditions, and working environment. Persons of high SES have the knowledge and adequate financial means, which allow them to maximally minimize the effects of various diseases, including allergic diseases.

The goal of the present study was to examine the dependence between respiratory allergy and quality of life (QoL) in young people and to assess whether the socio-economic status could modify this dependence.

2 Methods

The study protocol was approved by a local Ethics Committee. The study was conducted among students of the University of Opole and the Jagiellonian University in Cracow, Poland. In total, data were collected from 458 women and 363 men aged 18–25. All subjects were surveyed on their socio-economic status. The following variables were considered: the place of residence before the attending a college, the number of siblings, the parents' education, and the self-estimate of a financial situation of his/her

family. The place of residence was defined as a village, town – if fewer than 100 thousand inhabitants, and city – if over 100 thousand inhabitants. The parents' education was considered in three categories: vocational, secondary, and higher education, and the number of siblings in the categories of 0, 1, 2, or more. Financial situation was described by respondents based on the answers to the question: Is the material condition of my family below average, average, good, very good, difficult to identify, or variable. Since the last category was chosen by only two persons, it was omitted and the data of these two persons were discarded from further analysis. Taking into consideration all the socio-economic variables analyzed, the subjects were classified as being of low, average, or high status. The division was introduced on the basis of the value of the first component obtained in the principal components method (PCA).

Quality of life was based on the Polish version of the SF-36 test, which can subjectively evaluate the current physical and emotional state. The questionnaire includes 11 questions and 36 statements, by which the person surveyed determines the following:

1. General health
2. Physical functioning
3. Limitations due to physical health problems (Role-physical)
4. Limitations due to emotional health problems (Role-emotional)
5. Social functioning
6. Bodily pain
7. Vitality
8. Mental health

The sum of points enables the assessment of QoL. The sum of components 1, 2, 3, and 6 constitutes 'physical health', and the sum of components 4, 5, 7, and 8 constitutes – 'mental health'.

The results were normalized according to the formula:

$$\frac{(x - \min)}{(\max - \min)} \times 100$$

where x is the number of points received by a person; min – max is the minimum and maximum number of points possible to obtain on the scale. The calculation normalizes the scale range to 0–100, with 0 being the worst and 100 being the best possible score.

The occurrence of allergy was determined on the basis of answers to the questions whether the allergy and its specific allergens were defined on the basis of medical work-up.

The prevalence of allergy in different socio-economic groups was compared with a Chi^2 test. Differences in SF-36 scores in relation to the occurrence of allergy were assessed with a *t*-test. Differences in quality of life in relation to the occurrence of allergy and socio-economic status were tested with a two- factor analysis of variance. A $p < 0.05$ was considered as statistically significant.

3 Results

Respiratory allergy or respiratory and food allergy were declared by 88 (19.2 %) of women and 69 (19.0 %) of men. There were no significant differences in the incidence of allergies in relation to gender, whereas appreciable differences were noted depending on the socio-economic status. The prevalence of allergies was higher in the students of high SES than that in the low SES students (Table 52.1).

The results of the SF-36 test are shown in Table 52.2. The students suffering from allergic reactions obtained lower scores in terms of all domains of QoL, but the differences were small and statistically insignificant. However, these differences were cumulative, and the overall QoL index in the persons suffering from allergies was significantly lower than that in those who did not have allergic reactions (Table 52.2).

Among men, differences in QoL due to the presence of allergic reactions, were larger than those in women. The extent of these differences was also significantly associated with the socio-economic variables (Table 52.2). The results of multivariate analysis of variances for the

Table 52.1 Prevalence of allergy among students in relation to socio-economic status (SES)

		Allergy incidence		
		No	Yes	
SES Factor	Category	n (%)	n (%)	p
Mother's education	1. Vocational	197 (87.2)	29 (12.8)	$<0.01^{3}$
	2. Secondary	189 (81.5)	43 (18.5)	$<0.01^{1}$
	3. University	279 (76.7)	85 (23.4)	$<0.01^{2}$
Father's education	1. Vocational	220 (86.3)	35 (13.7)	$<0.05^{3}$
	2. Secondary	245 (81.1)	57 (18.9)	$<0.01^{1}$
	3. University	200 (75.5)	65 (24.5)	$<0.01^{2}$
Material conditions	1. Below average	190 (90.9)	19 (9.1)	$<0.01^{4}$
	2. Average	189 (81.8)	42 (18.2)	$<0.01^{1}$
	3. Good	183 (75.9)	58 (24.1)	$<0.01^{1, 2}$
	4. Very good	103 (73.1)	38 (27.0)	$<0.01^{2, 3}$
Place of living	1. Village	196 (88.3)	26 (11.7)	$<0.01^{3}$
	2. Cities <100,000 inhabitants	189 (82.2)	41 (17.8)	$<0.01^{1}$
	3. Cities >100,000 inhabitants	280 (75.7)	90 (24.3)	$<0.01^{2}$

P-values based on χ^2 test; Superscript by a p-value shows from which other category in a given socio-economic factor the category marked with the superscript is significantly different

Table 52.2 Quality of life in allergic conditions

	Females			Males		
	No	Yes	p	No	Yes	p
General health	66.49 ± 2.24	64.30 ± 2.43	NS	65.23 ± 2.61	62.30 ± 2.43	NS
Physical functioning	83.89 ± 2.17	79.71 ± 1.89	NS	84.01 ± 2.26	77.62 ± 2.03	NS
Role-physical	94.71 ± 3.43	89.02 ± 4.00	NS	95.00 ± 3.77	87.12 ± 3.84	NS
Role-emotional	78.11 ± 4.38	77.24 ± 4.12	NS	77.15 ± 4.17	75.15 ± 4.46	NS
Social functioning	81.17 ± 1.92	78.23 ± 1.89	NS	84.14 ± 2.03	77.43 ± 1.72	NS
Bodily pain	60.00 ± 4.19	58.05 ± 5.12	NS	62.00 ± 3.96	58.60 ± 5.23	NS
Vitality	58.16 ± 4.18	55.32 ± 4.13	NS	64.43 ± 4.00	58.30 ± 4.21	NS
Mental health	66.99 ± 3.41	62.62 ± 3.36	NS	68.01 ± 3.56	63.26 ± 4.06	NS
Sum of physical health	76.84 ± 4.15	66.80 ± 4.22	<0.05	75.69 ± 3.94	64.12 ± 4.87	<0.05
Sum of mental health	71.47 ± 3.62	62.91 ± 4.10	<0.05	75.32 ± 4.03	65.91 ± 4.10	<0.05
QoL	75.60 ± 2.78	63.86 ± 3.11	<0.01	77.12 ± 2.77	65.04 ± 2.62	<0.01

P-values based on *t*-test for the comparison between the presence and lack of allergy, *NS* nonsignificant

dependent variable 'QoL index' indicate that the pattern of differences in QoL between allergic and non-allergic subjects depended on the SES; the dependence was apparent in both physical and mental domains of health (Table 52.3). In case of low SES, the differences in QoL between the students suffering from allergy and those who did not have allergy were larger than in the groups with high SES. This concerned both women and men.

4 Discussion

Allergic reactions are often associated with pain ailments, notably of head or eyes, difficulties in breathing, or episodes of breathlessness. Frequent attacks of allergy undoubtedly impede the functioning in everyday life and relate to the emotional, vocational, and social sphere (Majani et al. 2001, 2003; Sicherer et al. 2001; Leynaert

Table 52.3 Dependence of quality of life (QoL) on the interaction between socio-economic status (SES) and the presence of allergy

			Females			Males		
SES factor	Category	Allergy	Physical health	Mental health	QoL	Physical health	Mental health	QoL
Mother's education	Vocational	No	76.79 ± 4.22	71.23 ± 3.78	75.00 ± 2.61	74.12 ± 2.99	76.43 ± 4.12	75.26 ± 3.12
		Yes	62.70 ± 4.62	60.56 ± 4.03	61.10 ± 3.14	62.05 ± 4.01	64.92 ± 3.56	62.98 ± 3.62
	Secondary	No	75.90 ± 4.15	70.96 ± 3.90	75.90 ± 3.23	75.03 ± 3.12	75.12 ± 3.69	77.14 ± 3.39
		Yes	65.72 ± 3.89	61.45 ± 3.73	63.54 ± 3.60	64.81 ± 3.63	65.01 ± 4.08	65.10 ± 2.98
	University	No	77.12 ± 4.30	72.15 ± 3.62	76.19 ± 2.96	76.89 ± 4.15	74.98 ± 4.15	77.96 ± 2.63
		Yes	69.00 ± 4.36	63.17 ± 4.11	64.69 ± 3.10	66.50 ± 3.01	65.89 ± 3.72	67.04 ± 3.12
p			<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Father's education	Vocational	No	75.84 ± 3.19	71.90 ± 4.53	73.81 ± 2.96	75.16 ± 3.33	75.37 ± 3.67	75.10 ± 2.76
		Yes	62.80 ± 4.37	61.89 ± 4.62	61.11 ± 3.10	62.10 ± 4.15	63.66 ± 3.46	62.12 ± 3.15
	Secondary	No	76.23 ± 4.69	71.23 ± 3.96	74.89 ± 2.87	74.89 ± 2.96	75.23 ± 4.15	75.14 ± 2.71
		Yes	67.92 ± 4.53	62.86 ± 4.41	64.54 ± 3.41	64.76 ± 4.01	64.92 ± 4.05	64.80 ± 2.82
	University	No	76.91 ± 4.26	71.86 ± 3.69	77.17 ± 2.62	77.01 ± 3.56	75.93 ± 3.92	76.85 ± 3.15
		Yes	70.91 ± 4.41	63.00 ± 3.66	66.69 ± 2.98	66.91 ± 3.49	66.01 ± 4.06	66.52 ± 3.24
P			<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Material conditions	Below average	No	74.22 ± 3.62	72.53 ± 3.62	73.12 ± 2.63	75.23 ± 2.63	75.43 ± 4.10	75.30 ± 3.15
		Yes	59.80 ± 4.97	62.82 ± 4.10	60.43 ± 3.21	61.42 ± 3.15	62.98 ± 4.00	61.95 ± 2.63
	Average	No	75.84 ± 4.18	71.47 ± 3.62	73.89 ± 2.66	75.29 ± 3.63	75.12 ± 3.68	75.15 ± 2.86
		Yes	65.80 ± 4.38	62.16 ± 4.10	63.06 ± 2.76	62.81 ± 3.85	64.63 ± 4.02	63.78 ± 3.62
	Good	No	76.12 ± 3.78	71.52 ± 3.62	75.52 ± 2.82	74.99 ± 2.69	75.86 ± 3.96	75.55 ± 3.65
		Yes	67.15 ± 4.64	63.00 ± 4.10	65.69 ± 3.01	64.55 ± 4.01	66.22 ± 3.93	65.52 ± 3.28
	Very good	No	76.15 ± 4.13	71.63 ± 3.62	76.74 ± 3.47	75.12 ± 3.99	75.88 ± 3.62	75.62 ± 3.46
		Yes	68.60 ± 4.56	63.12 ± 4.10	67.11 ± 3.11	64.99 ± 3.61	66.79 ± 3.77	65.92 ± 3.66
p			<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

P-values, based on MANOVA, indicate the significance of interaction between SES and allergy on a given domain of QoL

et al. 2000). When left untreated, allergic diseases have serious health consequences. Current medical knowledge enables to minimize the frequency of allergic episodes and the severity of symptoms. However, the quality of life in allergic diseases is not merely related with the absence of symptoms, but also with the emotions correspondent with the anticipated occurrence of an allergic episode. The fear resulting from the anticipation is usually the underlying mechanism of deterioration of quality of life, even if symptoms are not present (Farnik-Brodzinska and Pierzchala 2001).

Studies in children and adolescents with allergic rhinitis and bronchial asthma have shown that these diseases affect many aspects of daily functioning of patients, especially in the psychomotor sphere. There may develop inappropriate behaviors, irritability, frequent mood changes, and a decreased interest in school and social activities. Children affected with allergy exhibit a lower ability to learn and memorize new information and deterioration in perception in comparison with healthy children. In addition, children with allergy are prone to depression and have a sense of easy fatigue, even during relatively little efforts (Farnik-Brodzinska and Pierzchala 2002).

In adults reporting various symptoms of allergy, there has been an impairment of psychomotor skills, mainly eye-hand coordination, lack of concentration, and deficiency in decision making and other cognitive functions, clearly apparent during the time of symptoms relapse, e.g., in allergic rhinitis or conjunctivitis, compared with the asymptomatic time (Farnik-Brodzinska and Pierzchala 2002). Studies using the SF-36 test showed that allergy affects nearly all domains of quality of life (Kalpakhoglu et al. 2006; Bousquet et al. 1996).

In the presented study, persons affected with allergy scored lower in the SF-36 test than the healthy ones, but the statistically significant differences were revealed only in the total test result. In terms of specific, single aspects of QoL the differences were rather small, not reaching the level of significance. This might be due to the fact that persons affected with allergy were mostly coming with the higher rather than low socio-economic status; the finding being in accord with other studies on the subject (Gold 2006; Lewis et al. 2001; Strachan 2000; Heinrich et al. 1998; Ernst 1996). The influence of the socio-economic status on the biological condition or state of health is of an indirect nature. The status is a determinant of the conditions and way of life. Numerous studies have shown that the lifestyle of persons of high status reinforces the occurrence of allergy. This involves excessive hygiene or frequent stay in closed, air-conditioned rooms. Although persons of high status are more often affected with allergy, they likely have an adequate knowledge about the disease and the financial means that enable to minimize the effects of disease and the prevent its further episodes. Treatment of allergy by specialists significantly improves the quality of life outcomes (Bagenstose and Bernstein 1999; Bousquet et al. 1996).

In the present study, differences in quality of life due to the occurrence of allergy were much higher in persons of low status than in those of high status. Similar results have been obtained in the U.S. studies which show that allergies are less likely in people of low socio-economic status, but if allergy does exist in such a person, then it runs a stronger course compared with that in a person of high status (Lewis et al. 2001). Likewise, in studies conducted in 13 different countries, there was a significantly higher incidence of allergic symptoms among children from families of low status than among children from families of high status. These differences are largely the result of greater exposure of children from families of low status to risk factors, such as smoking during pregnancy, pet ownership, crowding, mould/moisture in the home, use of gas for cooking, or air pollution (Gehring et al. 2006).

A relationship has also been found between the socio-economic status, the presence of allergy, and the quality of life. A low level of education among patients suffering from allergy and asthma favors reduced quality of life, especially in persons with overweight and obesity in whom the incidence of overweight and obesity is inversely correlated with the level of education

(Kalpakhoglu et al. 2006). Moreover, in such persons, allergy often remains undiagnosed or treated. In persons of high status, on the other hand, despite a higher prevalence of atopy, one can rarely observe severe allergic episodes, which is likely due their being under constant medical care. This reasoning is confirmed by the studies that show that asthma, which often develops on the basis of allergy, is more common in persons with low socio-economic status (Ruijsbroek et al. 2011; Basagana et al. 2004).

There are some limitations of the present findings. The study included only students, who may not be a fully representative group of the general public. In addition, persons from families with very low income were not enrolled, since such persons tend to become quickly independent, leave school, and take up jobs at early age. Persons who were severely ill could not participate in the study either due to their health condition. On the other hand, however, testing just young students has some advantages. The quality of life is highly influenced by lifestyle and living conditions. Among students, there is a kind of equalization of the way of spending the leisure time, eating habits, and staying on campus, where they are equally exposed to risk factors for allergy. Thus, other stray factors that could influence the SF-36 test results concerning the influence of allergy on quality of life could be avoided.

In conclusion, the study demonstrates that the course of allergic diseases is highly dependent on the socio-economic status. The prevalence of allergy among students of low status is lower than that among students of high status. Nevertheless, allergy impairs the quality of life of low status students to a greater extent.

Conflicts of Interest No conflicts of interest were declared in relation to this paper.

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Exposure to Paracetamol and Antibiotics in Early Life and Elevated Risk of Asthma in Childhood

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Abstract

Prospective studies on increased risk of childhood asthma due to exposure to paracetamol and antibiotics in early life have yielded contradictory results. Therefore, the aim of the present study was to investigate the association between administration of paracetamol and antibiotics in the first 12 months of life and delayed asthma symptoms later in childhood. This is a cross-sectional study that included 1,063 children from the primary schools in Coimbra, Portugal. ISAAC-based environmental and core asthma and rhinitis questionnaires were used to obtain information about children's respiratory health and administration of paracetamol and antibiotics. We found that early paracetamol use significantly increased the risk of asthma ever (at least one episode in life) (OR = 2.9; 95 % CI:1.8–4.5), current asthma (OR = 2.4; 95 %CI:1.5–3.6), wheezing ever (OR = 2.5; 95 %CI:1.8–3.4), rhinitis ever (OR = 2.4; 95 %CI:1.7–3.3), and current rhinitis (OR = 2.8; 95 %CI:2.0–3.9). Antibiotic exposure showed a similar effect with the risk for current asthma (OR = 1.6; 95 %CI:1.0–2.5), asthma ever (OR = 2.0; 95 %CI:1.3–3.1), wheeze ever (OR = 2.3; 95 %CI:1.7–3.2), and rhinitis symptoms (OR = 1.8; 95 %CI:1.3–2.6, OR = 1.8; 95 %CI:1.3–2.6, OR = 1.9; 95 % CI:1.2–3.0 for rhinitis ever, current rhinitis, and tearing, respectively). We further found that increased frequency of paracetamol use during the last 12 months preceding the study facilitated the appearance of allergic symptoms, suggesting a dose-dependent associations. In conclusion, the study shows a significant association between exposure to paracetamol

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DOI 10.1007/978-94-007-6627-3_53,

and antibiotics in the first 12 months of life and both prevalence and severity of asthma and rhinitis symptoms in children 5–9 years old.

Keywords
Antibiotics • Asthma • Children • Paracetamol • Rhinitis • Wheeze

1 Introduction

A rapidly increasing prevalence of atopic diseases world-wide has led to a research interest in the possible underlying reasons (Eder et al. 2006), although a consensus regarding the asthmatic and allergic states has not yet been reached. On the list of putative factors increasing the prevalence of asthma is the early use of paracetamol and antibiotics (Rusconi et al. 2011).

Paracetamol was indicated as a candidate asthma facilitating factor in 1998, suggesting that the switch in the use pattern from aspirin to paracetamol in early childhood could increase susceptibility to allergic diseases by enhancing the Th2 response (Varner et al. 1998). Indeed, paracetamol is currently the most commonly used analgesic and antipyretic prescribed to pediatric patients as an alternative to aspirin that may be associated with the Reye syndrome (Newson et al. 2000; Rahwan and Rahwan 1986).

A large amount of epidemiological data demonstrating the role of paracetamol and antibiotic use in infancy for the development of asthma comes from the International Study of Asthma and Allergies in Childhood (ISAAC) (Foliaki et al. 2009; Beasley et al. 2008) or, like this work, from studies using the ISAAC tools (Barragan-Meijueiro et al. 2006).

A frequently described phenomenon justifying the rising prevalence of allergic diseases, including asthma, is the 'hygiene hypothesis', underlining the importance of microflora, especially gastrointestinal, in the development of a healthy immune system (Strachan 1989). Prescription of antibiotics applied in early life can alter microflora, prevent the boosting of immune system activity, and lead to increased allergic sensitization (Schaub et al. 2006). The perinatal period is characterized by the development of a Th1/Th2 balance, which could be distorted if exposed to antibiotic action (Prescott et al. 1999). Nevertheless, the results of studies are contentious. The ISAAC study resulted in many publications supporting the role of antibiotics in the development of atopic diseases. Data obtained from 193,412 children representing 29 countries show a significantly increased risk of allergic disorders in children who get antibiotics in the first year of life (Foliaki et al. 2009). There is increased risk for current asthma, asthma ever (at least one episode in life), severe asthma, or current rhinoconjunctivitis. Similar results were revealed for the cohort of 1,401 children from the US. This longitudinal study, initiated during pregnancy, show significantly higher risks for asthma and allergy, even stronger if the diagnosis takes place before the age of 3 (Risnes et al. 2011). On the other hand, no association between antibiotic and atopic diseases has been reported in 5 introspective studies in England (Celedon et al. 2002).

The objective of our study was to examine the association between antibiotics and paracetamol intake in the first year of life and the development of asthmatic symptoms in childhood.

2 Methods

The questionnaire was approved by the DGIDC (Direção-Geral da Educação) and the study had the approval of a local Ethics Committee.

This cross-sectional study evaluated 1,063 children from the first and second grade of primary school in the district of Coimbra, Portugal and was performed from September 2011 to June 2012. To assess data on respiratory conditions, the core ISAAC based asthma and rhinitis

questionnaires were used. For the analyses of the environmental and family factors, an ISAAC-based environmental questionnaire was applied. The questionnaires, translated to Portuguese, were used with the authorization of the national coordinator of the ISAAC initiative in Portugal. Parents filled out a questionnaire regarding the paracetamol and antibiotic exposure of children: In the first 12 months of your child's life, did you usually give paracetamol for fever? In the past 12 months, how often, on average, have you given your child paracetamol? In the first 12 months of life, did your child have any antibiotics?

Asthma ever was determined as a positive response to a question of a child ever having an asthma attack, and current asthma as at least one wheezing episode in the last 12 months of life or at least one night of cough attack not related to infection. Severity of the disease was estimated based on the responses to three questions about the number of wheeze attacks in the last 12 months, number of wake-up episodes caused by wheeze and problems in speaking caused by wheezing. Rhinitis ever was defined as ever having a problem of sneezing, running or blocked nose not caused by infections and current rhinitis as the same symptoms occurring in the last 12 months. Prematurity was defined as gestational age under 37 weeks.

Demographic data were presented in the percentage terms. Chi^2 test was run to calculate the prevalence differences between children exposed or not to antibiotics and paracetamol. Binary and Multinominal Logistic Regression were run to estimate the risk of atopic disease symptoms, depending on drug exposure, adjusted for children's age and sex, family history of asthma and rhinitis, and prematurity. Statistical software SPSS v 19 was used for data analysis.

3 Results

A total number of 1,063 questionnaires were returned by the parents of children. The children with severe chronic diseases not related to asthma or allergies were excluded from the study giving the final number of 1,037 children studied. The mean age of the children was 7.2 years (range 6–9 years) and 51 % of the children were girls, 49 % boys. Boys had a general tendency to have a higher prevalence of respiratory symptoms, although only current asthma prevailed significantly ($p < 0.05$). An attack of wheeze at least once in children's lives was reported by 35.2 % of parents and an attack during the last 12 months by 11.8 %. An asthma episode at least once in the child's lifetime was experienced by 10.4 %. A night cough attack was experienced by 24.3 % and exercise-induced asthma by 6.4 % children. Symptoms of rhinitis, at least once, were experienced by 22.8 % children during the last 12 months. Tearing and itchy eyes occurred in 9.6 %. Administration of paracetamol in the first year of life was reported in 21.3 % and antibiotics in 23.1 % children. In the last 12 months, 72.7 % of children were given paracetamol at least once and 12.7 % at least once per month. The full demographic characteristics can be found in Table 53.1.

Administration of paracetamol in early life significantly increased the prevalence of asthma ever (OR = 2.9; 95 %CI:1.8–4.5), current asthma (OR = 2.4; 95 %CI:1.5–3.6), wheezing ever (OR = 2.5; 95 %CI:1.8–3.4), rhinitis ever (OR = 2.4; 95 %CI:1.7–3.3) and current rhinitis (OR = 2.8; 95 %CI:2.0–3.9), episodes of itchy and tearing eyes (OR = 2.9; 95 %CI:1.8–4.5), and night cough attacks (OR = 3.1; 95 % CI:2.2–4.4) (Table 53.2). Among children who were given paracetamol in the first year of life, 53.6 % reported wheezing symptoms at least once later in life, compared with 30.8 % who did not receive the drug ($p < 0.01$). Not only the prevalence but also severity of symptoms appeared strongly associated with paracetamol administration in the first 12 months of life, such as the number of wheeze attacks in the last year (OR = 2.3; 95 %CI:1.4–3.8 for 1–3 attacks, OR = 2.7; 95 %CI:1.3–5.7 for 4 or more attacks), the number of wake-up episodes caused by the attacks (OR = 2.7; 95 %CI:1.5–4.9 for less than one per week and OR = 2.9; 95 % CI:1.2–7.0 for one or more per week).

Table 53.1 Demographic and prevalence data of the studied population

Variable	Yes (n)	Yes (%)
Sex F/M	529/508	51/49
Prematurity	191	19
Family history of asthma	427	41.5
Family history of rhinitis	445	43.5
Wheeze ever	357	35.2
Wheeze in the past 12 months	120	11.8
4 or more attacks of wheeze in the past 12 months	33	3.3
Sleep disturbance from wheeze, 1 or more nights a week in the past 12 months	23	2.3
Speech limited by wheeze in the past 12 months	18	1.8
Asthma ever	106	10.4
Wheeze during or after exercise in the past 12 months	65	6.4
Night cough in the past 12 months	248	24.3
Nose symptoms ever	233	22.8
Nose symptoms in the past 12 months	198	19.4
Nose and eye symptoms in the past 12 months	98	9.6
Nose symptoms affecting activities a lot in the past 12 months	4	2.0
Hay fever ever	13	1.3
Paracetamol intake in the first 12 months	218	21.3
Paracetamol intake at least once per month in the past 12 months	130	12.7
Paracetamol intake at least once the past 12 months	744	72.7
Antibiotic intake in the first 12 months	237	23.1
Number of participants	n = 1,037	

Prevalence of the factors included in this study was presented as the number and percentage of individuals declaring the existence of a factor

Table 53.2 Odds ratios (OR) for symptoms depending on paracetamol exposure in the first 12 months of life, with the level of significance (p) and confidence intervals (CI), both unadjusted and adjusted for sex, age, prematurity, and positive history of asthma and rhinitis

	Paracetamol exposure in the first 12 months of life					
	Unadjusted			Adjusted		
	OR	p	95 %CI	OR	p	95 %CI
Asthma ever	3.168	<0.001	2.074–4.838	2.851	<0.001	1.825–4.454
Current asthma (wheeze in the past 12 months)	2.615	<0.001	1.740–3.931	2.362	<0.001	1.532–3.642
Exercise induced asthma in the past 12 months	2.692	<0.001	1.598–4.534	2.392	0.002	1.381–4.143
Wheeze ever	2.598	<0.001	1.909–3.537	2.478	<0.001	1.794–3.422
Rhinitis ever	2.738	<0.001	1.974–3.799	2.372	<0.001	1.682–3.344
Current rhinitis	3.247	<0.001	2.311–4.562	2.754	<0.001	1.962–3.937
Night cough attacks in the past 12 months	3.427	<0.001	2.482–4.733	3.104	<0.001	2.205–4.370
Four or more wheeze attacks in the past 12 months	2.757	<0.001	1.345–5.652	2.711	0.008	1.286–5.717
One or more wake up episodes with wheeze/week in the past 12 months	2.642	<0.001	1.126–6.201	3.094	0.021	1.294–7.399
Nose and eye symptoms in the past 12 months	3.313	<0.001	2.143–5.124	2.876	<0.001	1.824–4.534

A similar association was found for antibiotics. The use of antibiotics resulted in increased risk of current asthma (OR = 1.6; 95 %CI:1.0–2.5), asthma ever (OR = 2.0; 95 % CI:1.3–3.1), wheeze ever (OR = 2.3; 95 % CI:1.7–3.2), night cough attacks (OR = 1.9; 95 %CI:1.3–2.6), and all rhinitis symptoms (OR = 1.8; 95 %CI:1.3–2.6, OR = 1.8; 95 %

Table 53.3 Odds ratios (OR) for each symptom depending on the antibiotic exposure in the first 12 months of life, with the levels of significance (p) and confidence intervals (CI) both unadjusted and adjusted for sex, age, prematurity, and positive history of asthma and rhinitis

	Antibiotic exposure by 12 months					
	Unadjusted			Adjusted		
	OR	p	95 %CI	OR	p	95 %CI
Asthma ever	2.094	0.001	1.364–3.216	1.963	0.003	1.251–3.081
Current asthma (wheeze in the past 12 months)	1.676	0.015	1.104–2.543	1.596	0.038	1.026–2.483
Exercise induced asthma in the past 12 months	1.537	0.125	0.888–2.659	2.465	0.266	0.780–2.465
Wheeze ever	2.292	<0.001	1.699–3.092	2.326	<0.001	1.702–3.180
Rhinitis ever	1.882	<0.001	1.357–2.611	1.836	0.001	1.302–2.589
Current rhinitis	1.884	<0.001	1.336–2.657	1.808	0.001	1.258–2.599
Night cough attacks in the past 12 months	1.909	<0.001	1.385–2.632	1.852	<0.001	1.315–2.608
Four or more wheeze attacks in the past 12 months	1.554	0.255	0.727–3.320	1.482	0.275	0.679–3.233
One or more wake up episodes with wheeze/week in the past 12 months	1.539	0.349	0.625–3.791	1.119	0.662	0.427–2.932
Nose and eye symptoms in the past 12 months	1.869	0.006	1.193–2.927	1.856	0.018	1.162–2.966

CI:1.3–2.6, and OR = 1.9; 95 %CI:1.2–3.0 for rhinitis ever, current rhinitis, and tearing and itching eyes, respectively). Although antibiotic intake was associated with the prevalence of asthma, it did not seem to influence its severity. We found no association between antibiotic intake and the number of wake-up episodes or speech problems, nor with the disturbance of daily activities caused by symptoms. A list of selected odds ratios was presented in Table 53.3.

We further found that increased frequency of paracetamol use during the last 12 months preceding the study facilitated the appearance of allergic symptoms, suggesting a dose-dependent associations. Here, risk of current asthma was OR = 3.3; 95 %CI:1.5–7.6, exercise-induced asthma (OR = 3.9; 95 %CI:1.2–12.1), night cough attacks (OR = 5.4; 95 %CI:2.9–9.9), current rhinitis (OR = 5.3; 95 %CI:2.7–10.5), and tearing and itchy eyes (OR = 6.7; 95 % CI:2.5–18.3). Finally, we found a strong association between the intake of paracetamol early in life and that during the last 12 months preceding the study; 30.6 % of the children who were given paracetamol in the first year of life also received paracetamol at least once per month during the last 12 months compared with just 7.8 % of those not having paracetamol exposure early in life ($p < 0.01$).

4 Discussion

The major finding of our study was that children exposed in early life to paracetamol or antibiotics appeared to have highly increased risk of developing asthma and rhinitis by the age of 5–9 and they also had more severe symptoms than those unexposed. We found positive associations between the use of paracetamol and antibiotics and the appearance of asthma ever or current asthma and rhinitis ever or current rhinitis. We also found an association between the dose-dependent current use of paracetamol and both prevalence and severity of current respiratory symptoms. Our results demonstrate that parents who gave paracetamol to their children early in life continue its administration later in life, and do so at higher doses. These associations remained strong after adjustments for sex, age, family history of asthma and rhinitis, and prematurity.

The present results are consistent with other epidemiological data, demonstrating an important role of antibiotics and paracetamol in the development of allergic diseases. A multicenter study on a sample of 205,487 children aged 6–7 from 31 countries, originating from the phase three of the ISAAC program, found that the intake of paracetamol in the first year of life increases the risk of

asthma, rhinoconjunctivitis, and eczema (Beasley et al. 2008). That study also verified the role of antibiotic intake in the prevalence of atopic diseases. Exposure to antibiotics in the first 12 months of life had a strong impact on future respiratory health. The reported use of antibiotics was strongly associated with increased risk of current asthma symptoms, severe asthma, and asthma ever and less, but still significantly, with current symptoms of rhinitis (Foliaki et al. 2009). These results were corroborated in other studies as well (Gonzalez-Barcala et al. 2012; Kozyrskyj et al. 2007).

The mechanisms behind these associations are unclear. Yet some plausible explanations could be proposed. Evidence exists that frequent increases of body temperature above 38 °C reduce the risk of asthma later in life (Williams et al. 2004). Therefore, antipyretic effects of paracetamol and antibiotics could reduce the protective role of fever, increasing the risk of asthma and allergies. Another theory suggests that the substitution of the anti-inflammatory ibuprofen for paracetamol has been a real cause of a higher allergic susceptibility. That claim is based on the fact that ibuprofen could have a protective effect against atopic diseases (Varner et al. 1998). Finally, paracetamol can lead to asthmatic inflammation by lowering glutathione level, which impairs the antioxidant defences (Rahman and MacNee 2000) and triggers inflammation through disruption of Th1-Th2 cytokine responses (Dimova et al. 2005).

'Hygiene hypothesis' (Strachan 1989) combined with 'antioxidant hypothesis' (Soutar et al. 1997) propose that decreased exposure to microbes and diet changes in the last decades (lower intake of antioxidants) could be responsible for a rapid increase in asthma prevalence in industrialized countries. Others also believe that administration of antibiotics early in life has a destructive effect on microflora, preventing the immunological system from correct maturation (Verhulst et al. 2008).

Despite the evidence from epidemiological studies, prospective research on the subject is less consistent. A birth cohort study including 16,933 6–7-year-olds from Italy analyzed the association between antibiotic and paracetamol use and asthma symptoms, using the ISAAC questionnaire. The focus was on two different phenotypes of asthma: early (first 2 years of life) and late onset of wheezing (during the last 12 months). A positive association has been confirmed only with the former (Rusconi et al. 2011), which is explained by the fact that early wheeze caused by viral infection is often misdiagnosed as asthma or allergy, and treated with paracetamol and antibiotics. Another cohort study of 198 children at high atopic risk also questions the true association between paracetamol and antibiotic use and asthma symptoms (Kusel et al. 2008). The authors reported that children who are given antibiotics in the first year of life have over twice the odds for asthma diagnosis, but this association became insignificant after adjustments for antibiotic predictor score, sex, first year pets, childcare, and general practitioner visits.

The above-mentioned inconsistencies are most frequently explained by 'reverse causation'. According to this assumption, children with respiratory disorders, including asthma, are more likely to be prescribed antibiotics and paracetamol (Kummeling and Thijs 2008). Following this reasoning, intake of drugs is a consequence rather than cause of atopic diseases. Conflicting results may be due also to another confounder – 'indication'. In infancy it is difficult to distinguish the allergic from viral or bacterial infection symptoms, which all are treated by paracetamol and antibiotics, and those infections could be a real underlying trigger for further development of asthma and allergies (Kummeling and Thijs 2008). However, studies on *in utero* exposure to paracetamol demonstrate increased risk of wheezing in children whose mothers received the drug, and such results are devoid of the 'indication' confounder (Bakkeheim et al. 2011).

5 Conclusions

Although the cross-sectional studies are prone to bias due to the lack of chronological information about the dosage and onset of symptoms, a strong association between early administration of antibiotics and paracetamol should not be

ignored. Further investigation should be focused on different phenotypes and epigenetic character of these associations. Unnecessary exposure to paracetamol and antibiotics, especially in infancy, should be avoided.

Acknowledgments Supported by a grant of the Fundação para a Ciência e a Tecnologia number SFRH/BD/66877/2009.

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

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Health Behaviors and Quality of Life Among Patients with Chronic Respiratory Disease

54

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Abstract

The purpose of this study was to analyze health behaviors of patients with chronic respiratory disease and the correlations between health behaviors (HB) and quality of life (QoL). The study involved 256 adult patients (135 women and 121 men), mean age was 62.6 ± 16.1 years, with chronic respiratory disease. The research tools consisted of the Health Behavior Inventory (HBI) and the World Health Organization Quality of Life Instrument Short Form. The mean general score for HB was 85.6 ± 17.8. Most patients (74 persons) obtained high scores (7–10 stens). The strongest correlations between QoL and HB were as follows: Psychological Domain correlated with positive mental attitude ($r = 0.308$, $p < 0.001$), healthy eating habits ($r = 0.224$, $p = 0.001$), and with the level of health behaviors ($r = 0.222$, $p = 0.003$); Social Relationship Domain correlated with positive mental attitude ($r = 0.282$, $p < 0.001$) and healthy eating habits ($r = 0.238$, $p < 0.001$). We conclude that QoL in patients with chronic respiratory diseases is significantly shaped by their health behaviors.

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_54,

Keywords
Chronic respiratory diseases • Health behaviors • Mental attitude • Quality of life

1 Introduction

An overall clinical status of patients with chronic respiratory diseases depends on many factors, including age and health behaviors (Andysz and Merecz 2012). An increasing importance is attributed to environmental, social, and behavioral factors, including health behaviors, which have an influence on quality of life and health state of chronically ill patients. Still, there are only few reports on health behaviors demonstrated by patients with respiratory disorders and the effects on their quality of life.

In patients with chronic respiratory diseases, quality of life is affected by physical and mental suffering associated with breathing problems, especially while doing everyday life activities. The group of chronic respiratory diseases accompanied by dyspnea is dominated by chronic obstructive pulmonary disease (COPD) and asthma (Soriano et al. 2005; Tinkelman et al. 2006). COPD is one of the most common respiratory diseases, and it is also the main cause of chronic morbidity and mortality in adult population. It is also the economic and social burden in the entire world. COPD is the fifth leading cause of death, and there are predictions that its incidence and COPD-related mortality rate will increase in future decades (Wen-Miin et al. 2008). Additionally, asthma is a chronic disease which is usually diagnosed and treated in primary care centers. Most patients are diagnosed as having mild or moderate asthma. A GP's practice shows that chronic respiratory diseases can be manifest by moderate symptoms, with no reflection in the disturbed lung function, increased daily variability of peak expiratory flow (PEF) values, or exacerbation of a disease (Ehrs et al. 2001; Chelminska et al. 2007). Therefore, it is important to apply methods which enable control and self-control over chronic diseases, and help determine quality of life and health behaviors of chronically ill patients. Health behaviors which are understood as actions adopted for health reasons, or ones with a documented influence upon health, are important elements of prevention and treatment in chronic respiratory diseases. These clinical developments result from harmful behaviors and practices (van Schayck and Chavannes 2003; Wen-Miin et al. 2008; Slusarska and Nowicki 2010).

The purpose of this study was to analyze health behaviors in patients with chronic respiratory diseases, and correlations between health behaviors and quality of life.

2 Methods

The study was approved by the Bioethical Commission of the Wroclaw Medical University (no. KB 608/2011).

The study group consisted of 256 adult patients with chronic respiratory diseases. The patients were diagnosed, according to ICD-10 as having: J45 bronchial asthma – 36.5 % (108), J42 unspecified chronic bronchitis – 15.5 % (46), J44 other chronic obstructive pulmonary diseases – 15.5 % (46), J43 pulmonary emphysema – 14.9 % (44), J41 chronic simple and mucous-purulent bronchitis – 12.8 % (38), J47 bronchiectasis – 4.7 % (14). Chronic respiratory diseases were accompanied by the following co-existing disorders (according to ICD–10): I10 essential (primary) hypertension – 27.7 % (82), M47 spondylosis – 26.0 % (77), I70 atherosclerosis – 21.0 % (62), E11 non-insulin-dependent diabetes mellitus – 12.5 % (37), I11 hypertensive heart disease – 11.8 % (35), M15

Table 54.1 Sociodemographic data of chronically ill patients

	n	%
Gender		
Women	135	52.7
Men	121	47.3
Place of residence		
Village	75	29.5
Below 5,000[a]	29	11.4
5,000–10,000[a]	12	4.7
10,000–50,000[a]	68	26.8
50,000–100,000[a]	22	8.7
100,000–200,000[a]	22	8.7
Over 200,000[a]	26	10.2
Education		
Primary	47	18.6
Vocational	70	27.7
Secondary	70	27.7
Post-secondary	39	15.4
Higher	27	19.7
Marital status		
Bachelor/maiden	31	12.2
Husband/wife	154	60.4
Divorcee	7	2.7
Widower/widow	63	24.7

[a]City population

osteoarthritis of multiple joints – 11.5 % (34), I25 chronic ischemic heart disease – 11.5 % (34). The mean age was 62.60 ± 16.14 years. The participants' sociodemographic data are shown in Table 54.1.

Participants for the study were recruited from patients of 130 general practitioners. GPs indicated patients with a diagnosis of chronic respiratory disease. The project staff members asked randomly chosen patients if they would like to participate in the study. Patients who agreed to take part anonymously in the project signed an informed consent form. The patients were given a questionnaire to complete at home and return in a stamped envelope.

In the study, two research tools were used, namely the Health Behavior Inventory (HBI) and the World Health Organization Quality of Life Instrument Short Form (WHOQOL-BREF). The Health Behavior Inventory (Juczynski 2001) allows for analyzing how often health behaviors (HB) are exhibited in four health-related categories: healthy eating habits (HEH), preventive behaviors (PB), positive mental attitude (PMA), and health practices (HP). The assessment was done with a five-point scale (from 'almost never' to 'almost always'). A general score for health behaviors was obtained through the sum of individual elements (24–120 score), where the score correlated with the intensity of healthy behaviors (the higher the score, the more healthy behaviors). Next, the raw data were converted into sten scores. There are no reference norms for the evaluation of four types of activities associated with healthy behaviors. However, it is possible to measure the intensity of health behaviors in particular categories; a result being the sum of all answers on a particular subscale should be divided by six (Andruszkiewicz and Basinska 2009).

The WHOQOL-BREF is an instrument for measuring quality of life within four domains (D1. Physical: Activities of daily living, Dependence on medicinal substances and medical aids, Energy and fatigue, Mobility, Pain and discomfort, Sleep and rest, Work capacity; D2. Psychological: Bodily image and appearance, Negative feelings, Positive feelings, Self-esteem, Religion/Spirituality/Personal beliefs, Thinking/learning/memory/concentration; D3. Social relationship: Personal relationships, Social support, Sexual activity; D4. Environmental: Financial resources, Freedom/physical safety and security, Health and social care: accessibility and quality, Home environment, Opportunities for acquiring new information and skills, Participation in and opportunities for recreation/leisure, Physical environment (pollution/noise/traffic/climate) and Transport). The WHOQOL-BREF is a shorter version of the WHOQOL-100, developed by the WHO group for the Research on Quality of Life (Dudzinska et al. 2011). The reliability of the Polish version of the WHOQOL-BREF questionnaire, assessed with the α-Cronbach coefficient, is very high, which refers both to its parts measuring particular domains (results from 0.81 to 0.69) and the questionnaire as a whole (0.90) (Bousquet et al. 1994; Jaracz et al. 2006).

R 2.10.1 (for Mac OS X Cocoa GUI) was used for statistical analysis. The distribution type for all variables was determined with a Shapiro-Wilk test. The critical level of significance was assumed at $p < 0.05$. For measurable (quantitative) variables arithmetic means (M) ± SD were calculated, while for qualitative variables, the frequency (percentage) was determined. The analysis of qualitative variable was based on contingency tables and the Chi^2 test. The Spearman rank correlation test was used to check the relations between variables. For each variable pair, the Spearman rank correlation coefficient was calculated, and the level $p < 0.05$ was assumed as statistically significant. Overall results of the Health Behavior Inventory (HBI) and the WHOQOL-BREF did not have normal distribution, which was confirmed by the Shapiro-Wilk normality test.

3 Results

In the general HB assessment, patients achieved the mean score of 85.6 ± 7.8. The results after converting the scores into stens were as follows: 40.9 % (74) of patients obtained high scores (7–10 stens), 34.3 % (62) medium scores (5–6 stens), and 24.9 % (45) low scores (1–4 stens). Healthy behaviors were found more common among women (56.5 %, 52 vs. 40.4 %, 36, $p = 0.044$), older patients ($r = 0.191$, $p = 0.010$), and those visiting a GP at least six times during the last 12 months (52.9 %, 54, vs. 41.9 %, 31, $r = 0.274$, $p < 0.001$). The intensity of HB depended also on the number of hospital stays during the last 3 years (M = 2.0 ± 3.9), and was higher in patients hospitalized at least once during the last 3 years (51.5 %, 52 vs. 45.1 %, 37; $r = 0.195$, $p = 0.008$).

The intensity of health behaviors within the four categories was calculated using average scores in all categories as indicators. The detailed analysis of health behaviors in the study group revealed that the most common were: preventive behaviors (M = 3.8 ± 1.3), positive mental attitude (M = 3.6 ± 0.9), health practices (M = 3.6 ± 1.0), and healthy eating habits (M = 3.3 ± 0.9). Preventive behaviors correlated with gender ($r = -0.139$, $p = 0.043$), being more common among women (51.9 %, 56, vs. 46.5 %, 46), and with age ($r = 0.184$, $p = 0.008$), being considerably more often observed among old patients (56.0 %, 61, vs. 41.7 %, 40). It was also noticed that BMI > 30 (48.1 %, 13) correlated with the intensity of preventive behaviors ($r = 0.240$, $p = 0.040$). The intensity of preventive behaviors was higher in patients visiting a GP at least six times during the last 12 months (54.9 %, 67 vs. 41.2 %, 33; $r = 0.227$, $p = 0.001$), and hospitalized at least once during the last 3 years (54.1 %, 60 vs. 43.9 %, 43; $r = 0.185$, $p = 0.007$). Positive mental attitude was more common among women (54.5 %, 60 vs. 40.0 %, 40, $p = 0.049$), and patients visiting a GP at least six times during the last 12 months (54.9 %, 67, vs. 36.6, 30; $r = 0.206$, $p = 0.003$). Health practices correlated with age ($r = 0.409$, $p < 0.001$), with BMI ($r = 250$, $p = 0.046$) and were significantly more often adopted by older patients (53.6 %, 52, vs. 28.4 %, 25). Health practices were also more popular among patients visiting a GP at least six times during the last 12 months (51.0 %, 53, vs. 30.8; $r = 0.249$, $p < 0.001$), and hospitalized at least once during the last 3 years (51.0 %, 53 vs. 31.8 %, 27; $r = 0.309$, $p < 0.001$). Healthy eating habits were significantly more common in women (52.7 %, 58 vs. 34.7 %, 35, $p = 0.012$; $r = -0.227$, $p < 0.001$) and patients visiting a GP at least six times during the last 12 months (44.0, 55 vs. 42.5, 34; $r = 0.157$, $p = 0.024$). Correlations within the HBI are shown in Table 54.2.

The strongest correlations were found between the Psychological Domain and positive mental attitude ($r = 0.308$, $p < 0.001$), and between the Social Relationship Domain and positive mental attitude ($r = 0.282$, $p < 0.001$). The analysis suggests that there were correlations between the Physical Domain and health practices ($r = -0.263$, $p < 0.001$); the Psychological Domain and healthy eating habits ($r = 0.224$, $p = 0.001$), the general level of health behaviors ($r = 0.222$, $p = 0.003$) and preventive behaviors ($r = 0.149$, $p = 0.031$); the Social Relationship Domain, healthy eating

Table 54.2 Correlations within the Health Behavior Inventory

Variables	r	P
HB vs. PMA	0.851	$p < 0.001$
HB vs. PB	0.845	$p < 0.001$
HB vs. HP	0.722	$p < 0.001$
PB vs. PMA	0.639	$p < 0.001$
HEH vs. PB	0.616	$p < 0.001$
HB vs. HEH	0.616	$p < 0.001$
HEH vs. PMA	0.587	$p < 0.001$
PB vs. HP	0.583	$p < 0.001$
PMA vs. HP	0.559	$p < 0.001$
HEH vs. HP	0.441	$p < 0.001$

r Spearman's rank correlation, *HB* health behaviors in general, *PMA* positive mental attitude, *PB* preventive behaviors, *HP* health practices, *HEH* healthy eating habits

habits ($r = 0.238$, $p < 0.001$), and the general level of health behaviors ($r = 0.159$, $p = 0.031$).

4 Discussion

The present study, measuring the influence of health behaviors on quality of life among patients with chronic respiratory diseases, is one of the very few in this field. Searching for the relationship between quality of life and health behaviors may contribute to the planning of therapy and rehabilitation for chronically ill patients. The literature on the subject provides information mainly about the research of health behaviors demonstrated by healthy people, whose results are used to design education programs. The comparison between individuals in good physical shape and those with chronic diseases shows that the latter ones more often demonstrate healthy behaviors, because they are aware of their importance for the therapeutic process.

In our study, the mean score for general assessment of health behaviors (HB) was high, which shows that the surveyed patients exhibited healthy behaviors. However, one-fourth of the surveyed did not have healthy behaviors, which was reflected by low scores (1–4 stens). Preventive behaviors, positive mental attitude, and health practices received the highest scores in the study group. The factors significantly contributing to adopting healthy behaviors were: female gender, old age, a higher number of visits to a GP in the last 12 months, and the number of hospital stays during the last 3 years. Slusarska and Nowicki (2010) and Binkowska-Bury et al. (2010) also showed that health behaviors are related to gender. Kurowska and Bialasik (2009) showed that chronically ill patients had a higher rate of healthy behaviors than those admitted to the hospital due to acute states. In a study conducted by Slusarska and Nowicki (2010) individuals who are potentially healthy and professionally working achieved medium scores (5–6 stens). As in our study, the categories with the highest evaluation were positive mental attitude and preventive behaviors. The lowest scores were noted in health practices (Slusarska and Nowicki 2010), which is discrepant with the present our results. This discrepancy may result from the participants' health state; chronically ill patients know that healthy behaviors improve their quality of life. Kurowska and Bialasik (2009) drew attention to the fact that patients began using health practices associated with rest, a regular sleep schedule, body mass control, limiting cigarette smoking, and avoiding physical overexertion. This was a consequence of the worsening health state and symptoms, which were becoming more severe with the progress of a disease (Kurowska and Bialasik 2009).

5 Conclusions

Quality of life among people with chronic respiratory diseases is significantly shaped by their health behaviors. The strongest correlations were observed between quality of life within the Psychological Domain and the Social Relationship Domain and such health behaviors as positive mental attitude, general level of health behaviors, and healthy eating habits.

Conflicts of Interest The authors have no financial or other relationships that might lead to conflicts of interest.

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Attitudes Toward Euthanasia Among Polish Physicians, Nurses and People Who Have No Professional Experience with the Terminally Ill

55

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Abstract

Euthanasia is an issue that generates an extensive social debate. Euthanasia is generally classified as either active or passive. The former is usually defined as taking specific steps to cause the patient's death, while the latter is described as withdrawal of medical treatment with the deliberate intention of bringing the patient's life to an end. The dispute on euthanasia involves a multitude of aspects including religious, legal, cultural, ethical, medical, and spiritual issues. The purpose of the present study was to examine the views of medical professionals toward the highly controversial issue of euthanasia. Accordingly, the research has been conducted among a group of Polish nurses and physicians working in Intensive Care and Oncology Units. Their views have been compared to those of the control group, which included the members of the general public, who do not work in medical profession. It was expected that the education and training and the day-to-day exposure to vegetative patients might influence the views of medical personnel concerning euthanasia. The research demonstrated that the members of all groups supported liberal views. Conservative views were not popular among the respondents. The physicians turned out to be the least conservative group. The survey has also demonstrated that there is a broad consensus that informational and psychological support should be provided to terminally ill patients and their relatives. The attitude toward the passive form of euthanasia seems to have

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DOI 10.1007/978-94-007-6627-3_55,

broad support. In particular doctors tend to approve this form of bringing a terminally ill patient's life to an end. The active euthanasia is regarded with much less favor and physicians, in particular, appear to disapprove of it.

Keywords
Attitudes • Euthanasia • Nurses • Palliative care • Physicians

1 Introduction

The term Euthanasia derives from the Greek *euthanatos*, which can be translated as 'a good death'. More specifically, it can be defined as a process aimed at causing the patient's death, with a view to minimizing his or her suffering. Within this category a distinction is often drawn between active and passive euthanasia (Reichenberg 1987). The difference is that while the former requires a positive act on the part of a person performing euthanasia, the latter is based on withdrawing or withholding a life-sustaining treatment. It appears to be fair to argue that, in general, passive euthanasia enjoys more support than active euthanasia. Both types of euthanasia, however, are highly controversial issues. Opponents criticize them as highly immoral and contrary to the public interest. Euthanasia is also contrary to the views of numerous major religions. On the other hand, proponents of euthanasia tend to argue that the individual freedom should extend to the level at which one is able to decide whether or not he would like to have his life sustained, where no hope of recovery exists and considerable suffering is involved.

The problem becomes more important as progress in medical science makes it possible to keep terminally ill patients and people in persistent vegetative state alive for many years (Yun et al. 2011). Mass media present numerous instances of such a dramatic cases. This includes those of Eluana Englaro, Terry Schiavo, and Diane Pretty. The example of Diane Pretty in the UK involved a woman suffering from a terminal disease attempted to influence a change in the legislation concerning euthanasia in her country. Her appeals to the public, in which she proclaimed: 'I want to have a quick death without suffering, at home surrounded with my family' did not persuade lawmakers (MacDonald 2008). The British Courts refused to recognize a right to die. Many people share this opinion, believing that life is of supreme value, and that no one should be allowed to assist another in committing suicide. Those who oppose this view, on the other hand, emphasize that the value of life cannot be isolated from the issue of quality of life. No satisfactory solution of the matter in dispute has been reached to date. However, this problem should be examined and solved very quickly, because it is a source of suffering for patients or for their relatives and friends.

The moral, philosophical, and religious controversy over euthanasia is also reflected in legislation. There are only three countries in Europe that allow active euthanasia: Belgium, Luxemburg, and the Netherlands. They are followed by Switzerland and, more recently by Germany, which permit an assisted suicide. In other European countries such as the UK, France, Ireland, and Poland, active euthanasia constitutes a criminal offence, but passive euthanasia is permissible in some circumstances. It is also worth noting that in many of the countries the anti-euthanasia laws are not fully enforced and that despite its potential for punishment, prosecutions are not always brought up (Griffiths et al. 2008).

Many studies have been conducted to explore perspectives on euthanasia and its legalization. The main participants of the studies were medical students and medical practitioners, who observe life-threatening events suffered by patients (Cuttini et al. 2004; Beder et al. 2010). Different results have been achieved dependent on cultural factors, systems of values,

knowledge, and the legal status of euthanasia (Cuttini et al. 2004; Iglesias et al. 2011). Generally, attitudes toward euthanasia are more positive in those countries where voluntary euthanasia is permitted, such as the Netherlands and Belgium. A survey conducted among Belgian medical personnel demonstrated that a broad spectrum of attitudes exists toward euthanasia among medical practitioners. The respondents were then divided into several sets. The first group included 23 % of all palliative care physicians and nurses who took part in a survey. The members of this group were convinced that euthanasia should be avoided as far as possible. However, they were unwilling to fully exclude voluntary euthanasia as a possibility in extreme circumstances. They also generally agreed that euthanasia was made superfluous by palliative sedation. Participants of a second group (35.2 %) were identified as 'moderate advocates of euthanasia'. Those who were included in this group had significant doubts regarding the appropriateness of voluntary euthanasia in particular cases, such as in under aged and demented patients. However, in general they favored a broader application of voluntary euthanasia. The third and largest group (41.8 %) of all respondents exhibited the most liberal attitude toward voluntary euthanasia. They only barely wanted to fully exclude the possibility of euthanasia in the case of minors or demented persons. Most of them were against non-voluntary euthanasia, but a majority of the group held a positive view of a law regulating non-voluntary euthanasia (Broeckaert et al. 2010).

Extremely different attitudes toward euthanasia were revealed in Pakistan. Around 60 % of participants saw euthanasia as murder and over 70 % considered it to be unethical. The right to die based on the patient's wish was respected by 16.2 % of medical students and 5.8 % of practitioners. The legalization of euthanasia in Pakistan was favored by 15.4 % of practitioners, compared with 10.4 % of students. The study also revealed that nearly half of the participants would not trust doctors if euthanasia became legal. Both students and doctors were strongly opposed to the performance of euthanasia in their country in the future (Hasan et al. 2012). Meade (1992) emphasized that physicians, nurses, and students of medical sciences have studied idealistic ethics of care, but in the workplace they make decisions that did not always reflect the theoretical ethics and once confronted with the issue in practice they do not always follow those ideals. Research indicates that more than half of Swedish oncologists declare they have heard their patients expressing a wish to pass away on their own. About one-third of them have provided the drugs or medicines in such doses that some of their patients' deaths were hastened (Valversius et al. 2000). Interesting data concerning euthanasia can be found it a report prepared by Dutch scientists, which includes an evaluation of the procedure. In the Netherlands, physicians must report euthanasia and physician-assisted suicide to enable a review by one of five regional multidisciplinary review committees. In this way it is possible to collect data about the motives of patients who request active euthanasia. The research indicates that 'physicians reported that the patient's request was well-considered because the patient was clear-headed (65 %) and/or repeated the request several times (23 %). Unbearable suffering is often substantiated with physical symptoms (62 %), loss of ability (33 %), dependency (28 %), or deterioration (15 %). In 35 % of the cases, physicians report that there have been alternatives to relieve patients' suffering, however the patient refuses to accept proposed form of treatment (Buiting et al. 2009).

Although most studies have focused on attitudes toward active euthanasia, some also explore beliefs about passive euthanasia. One issue raised by Broeckaert et al. (2010) in their research conducted in Belgium has been: 'In my opinion, there is a huge ethical difference between, on the one hand, withholding life-prolonging treatment (e.g., artificial nutrition and hydration), and, on the other hand, active termination of life (non-voluntary euthanasia) and (voluntary) euthanasia'. Over 90 % of nurses and 80 % of physicians studied have agreed with this opinion. The problem of euthanasia has attracted a large amount of attention around the

world. For this reason, study dedicated to this issue in Poland might also produce interesting results. The authors of the present article were particularly interested in whether there would be a uniformity between the doctrine of the Catholic church, which condemns euthanasia, and the personal views of respondents, in particular bearing in mind the fact that almost 90 % of Poles declare themselves as Catholics.

2 Methods

The Institutional Board for Human Research approved this survey study. The study consisted of two separate stages. In the first stage, the questionnaire called Attitude Toward Euthanasia was developed *de novo* by A. Glebocka and A. Gawor, the coauthors of this article. The development and verification of the questionnaire was carried out in a group of 108 randomly selected lay persons of different age and profession: 44 men and 64 women, mean age of 36 $\pm$ 12 years. The questionnaire consisted of 46 statements. Although the tool's reliability was deemed satisfactory (Cronbach's alpha = 0.83), it appeared appropriate to carry out a factor analysis (Varimax standardized) the pertinence index and to simplify the structure of the method. As a result, three components (factors) were distinguished, accounting for a total of 16.7 % variance. The first one, encompassing 12 statements, was called the informational support component; the second one consisted of 9 statements and was referred to liberal attitudes toward euthanasia; and the third one included 7 items designed to measure traditional and conservative attitudes. Examples of the items include statements such as: Factor I – (1) At every stage of treatment, patient's relatives should have access to psychological support; (2) Relatives should be given the opportunity to say goodbye to the dying patient; Factor II – (1) Each individual has the right to decide whether he or she wishes to continue living or not; (2) Where medicine is helpless and there is no hope of recovery, the patient's death should be facilitated; Factor III – (1) No one has the right to decide whether he or she wishes to continue living or not; (2) A patient's life should be sustained at any cost and regardless of suffering to the patient involved.

In the second stage of the study, the questionnaire developed as above outlined was applied to 92 participants: 27 physicians and 34 nurses working in Intensive Care and Oncology Units and 31 people uninvolved with medical profession; mean age of 36 $\pm$ 10 years. Almost 17 % of the participants were caring for disabled relatives.

Data collected from the Attitude Toward Euthanasia Questionnaire in the form of raw scores were analyzed using a multivariate analysis of variance (MANOVA). Statistically significant differences were set at $p < 0.05$.

3 Results

Two different grouping factors were applied to the multivariate analysis of variance of the results of the Attitude Toward Euthanasia Questionnaire: (1) grouping factor based on participants' profession (physicians vs. nurses vs. control group) – there were significant differences between the groups, Lambda Wilksa = 0.837, F (6.154) = 2.376 ($p < 0.5$); (2) grouping factor: looking after handicapped relatives (family caregivers vs. people having no experience with the terminally ill) – there were no significant main effects between the groups: Lambda Wilksa = 0.960, F(3.78) = 1.056 ($p > 0.05$).

The *post hoc* Scheffe's test showed significant differences among the mean scores of the three factorial subclasses of the questionnaire: informational, liberal, and conservative, as outlined in the methods above, without regard to occupational affiliation. All three factor-groups, however, strongly agreed that informational and psychological support should be provided, to both patients and their relatives. Moreover, in each of the factor-group, liberal attitudes were stronger than the conservative ones. Interestingly, the research also demonstrated the least support for the conservative views among the physicians; this result was appreciable different from that in the control group (Table 55.1).

Table 55.1 Attitude towards euthanasia questionnaire

	Physicians	Nurses	Controls
Factor I-Informational support	4.45 ± 0.51	4.56 ± 0.28	4.26 ± 0.63
Factor II-Liberal attitudes	3.54 ± 0.66	3.54 ± 0.70	3.41 ± 0.65
Factor III-Conservative attitudes	2.18 ± 0.37*	2.35 ± 0.56	2.63 ± 0.54

Values are means ± SD; *$p < 0.05$ for the difference between physicians and controls

Table 55.2 Attitude Toward Euthanasia Questionnaire: mean results for particular items – comparison between physicians and control group

Items of Attitude Towards Euthanasia Questionnaire	Physicians	Controls
If a terminally ill patient requests that the life sustaining treatment be withheld, his request should be followed in a manner that would enable him to die in an immediate and painless way.	2.00 ± 0.29*	3.11 ± 0.26
If a terminally ill patient requests that the life sustaining treatment be withheld, his request should be followed and one should let him die.	4.09 ± 0.24*	3.18 ± 0.22
A patient's life should be sustained, regardless of the pain to the patient involved.	1.31 ± 0.19**	2.59 ± 0.17
Families looking caring for their loved ones who are in vegetative state receive a lot of support from the state.	1.42 ± 0.20*	2.07 ± 0.18
Where medicine is helpless and there is no hope of recovery, the patient should be allowed to die.	4.04 ± 0.23*	3.11 ± 0.20
Not all people are capable of understanding the information on the need to withhold treatment.	4.40 ± 0.17*	3.85 ± 0.15
Religious believers should not impose their views on life and death upon others	4.72 ± 0.19*	4.03 ± 0.17
A patient's life should be sustained, regardless of the social and economic costs.	1.40 ± 0.26**	2.96 ± 0.23

Values are means ± SD; *$p < 0.05$; **$p < 0.001$ for differences between physicians and controls

Analysis of the mean results for particular items in the questionnaire indicated significant differences between the physicians and the control group (see Table 55.2). The research demonstrates that, when compared with the control group, the physicians are supportive of passive euthanasia. They are, however, much less in favor of active euthanasia. In so far as the active euthanasia is concerned as many as 65.38 % of physicians oppose it, while only 25 % members of the control group share such views. Active euthanasia is supported by 23 % of the physicians and 35.72 % of the control group participants. By contrast, when it comes to passive euthanasia, 7.7 % of physicians oppose it, and in the control group this number was 21.43 %. Support for this type of euthanasia amounts to 69.23 % for the physicians and 39.29 % of the control group.

4 Discussion

The aim of the present study was to investigate whether the views of professional medical personnel can be distinguished from those of people from other backgrounds. It was assumed that such differences might occur for a variety of reasons, most importantly because professional medical personnel have specific education and training and due to the fact that they have day-to-day exposure to patients in a vegetative state. The research demonstrates that medical staff had a mildly positive attitude toward the liberal views, and that support for liberal views among the control group was at approximately the same level (Yun et al. 2011). Regarding conservative attitudes, all the groups had limited sympathy for such views. In particular, the physicians turned out to have the least conservative attitudes.

All of the groups favored providing passive euthanasia. This type of euthanasia was supported by the physicians in particular. Support for active euthanasia was more limited (Beder et al. 2010). Members of the control group approved of this type of euthanasia, but to a much lesser extent than the passive one. A noticeable difference between the two types of euthanasia occurred among the physicians.

This group was more favorable towards passive euthanasia, but strongly opposed active euthanasia. The research also demonstrates strong and uniform support for the notion of providing terminally ill patients and their relatives with informational and psychological support.

The authors of this article are aware that the groups, however adequate for the present study, are not representative of the whole population of Poland. For that reason, a more general study on the views of the public would be welcomed. Moreover, it seems that investigating the causes for physicians' rejection of active euthanasia demands further research. This issue seems to be especially interesting when one takes into account the fact that this group represented itself as the one that is least influenced by religion in their attitudes towards euthanasia. Finally, the authors believe it would be interesting to see if the age or the area of specialization of the physician has a significant influence on their views. The intended scope of the present research did not allow this to be explored, but it is the authors' hope that the influence of such factors will be investigated in subsequent studies.

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

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Death as a Result of Violent Asphyxia in Autopsy Reports

56

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Abstract

Violent asphyxia can be subdivided into various kinds according to the mechanism, so that the resuscitation techniques are different in each case. The purpose of the present article was to analyze the autopsy reports of the Department of Forensic Medicine of the Medical University in Wroclaw, Poland of 2010, in which the established cause of death was violent asphyxia. We found that among the 890 autopsies performed, there were 164 cases of death due to violent asphyxia caused by drowning, choking on food, gastric fluid, or blood, hanging, manual strangulations, immobilization of the chest (positional asphyxia), environmental asphyxia due to substitution of the oxygen-rich air for some other gas, and others. The most common cause of death in the group was hanging, mostly suicidal hangings of alcohol-intoxicated males. Despite an early medical treatment consisting of removing the noose from the neck and suction the fluids from the mouth and bronchial tree to safe the central nervous system from imminent hypoxia, there were negative outcomes in most cases due to the development of critical brain ischemia, with deaths followed after several days spent in the intensive care units. No connection to gender or age of the deceased was noted. We conclude that violent asphyxia remains to be a quite commonly cause of death in the practice of forensic pathologists – among all the autopsies performed in 2010 every sixth was of an asphyxia victim.

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3_56,

Keywords
Choking • Drowning • Forensic medicine • Hanging • Violent asphyxia

1 Introduction

Violent asphyxia, in the broad sense, constitutes a quite common cause of death in the practice of forensic medicine. According to DiMaio and Dana (2006) and DiMaio and DiMaio (2000) death because of asphyxia can especially be precipitated by suffocation, strangulation, neck holds, chemical asphyxia, and drowning. Suffocation can be further subdivided into environmental suffocation, smothering, choking, traumatic, and mechanical asphyxia. Strangulation, in turn, can be subdivided into hanging, autoerotic hanging included, ligature strangulation, and manual strangulation. The resuscitation techniques applicable are different in each case. The purpose of the present study was to analyze retrospectively the autopsy reports made out by a department of forensic medicine during 1 year, in which the established cause of death was violent asphyxia.

2 Methods

The study was approved by a local Ethics Committee. Autopsy reports of the Department of Forensic Medicine of the Medical University in Wroclaw, Poland of 2010 were analyzed. Only were the reports taken into consideration in which the cause of death due to violent asphyxia was clearly established and the basic circumstances leading to death were known.

3 Results

In the year 2010, among the 890 autopsies performed, there were 164 cases when the established cause of death was violent asphyxia of some kind. The group was made up by 131 males and 33 females. The mean age of male victims was 44.7 years and that of female victims was 58.7 years. There were 37 cases of drowning, 28 cases of choking on food, gastric fluid, or blood (also resulting from complications of surgical procedures that were performed earlier), 80 cases of hanging, 2 cases of ligature and manual strangulations, and 1 case of immobilization of the chest causing positional asphyxia. There were 2 cases of environmental asphyxia because of removal of the oxygen-rich air by some other gas, propane-butane in the case of apparent cigarette lighter inhalation and helium in the probably autoerotism-related death, and using up the oxygen in a limited space by the victim with plastic bag put on the head. There were 2 cases of overtly iatrogenic origin, including choking with blood after uvulopalatoplasty due to throat cancer and due to post-operation regional edema.

In three cases, there was apparent concurrency of the causes of death when drowning coexisted with liver rupture in a victim during a flood time and when dental prosthesis got stuck in the throat of another victim blocking the airways during heart attack, and when drowning coexisted with choking on gastric fluid or mud. The most common cause of death in the group analyzed was hanging, mostly suicidal hangings of alcohol-intoxicated males. There were also some cases of deaths due to autoerotic asphyxia gone wrong.

When the time of death was considered, the incidence of drowning was the highest in the spring and summer months – starting from April (3 cases), May and June (4 cases), peaking in July (9 cases) and August (10 cases); in the remaining month not exceeding two cases per month. Deaths because of choking did not show any significant connection with the time of the year, their incidence ranging from 1 to 4 per month. The hangings happened most often in the months between April and November, with

the highest number in August (10 cases) and lowest in December (3 cases).

Concerning the asphyxia-related cases in which the level of alcohol was measured in blood samples collected during autopsy, 10 drowned victims were sober as compared with 27 who were intoxicated at the time of death. In the group of established hanging cases, this ratio was 35–45, respectively. Among the victims who died because of various kinds of choking, 17 were sober and 11 were intoxicated. In case of strangulation, one victim was sober and another one was intoxicated.

4 Discussion

Often the mechanism and manner of asphyxia-related death is initially, in the autopsy report, not fully determined, as it depends not only on the autopsy findings, but also on other determinants set by the police and attorney in a given case, but often withheld from, or unknown to, forensic medicine experts who perform the autopsy. This limitation alters the statistics presented in the study and limits the results to those cases in which the mechanism and manner of death were determined on the basis of the information available at the moment of completing the autopsy report. In 10 cases, the autopsy findings were suggestive of some kind of violent asphyxia, but more additional information from the attorneys was needed to further elaboration of death reasons, so that these cases were not sufficiently explained at the moment of forming the autopsy reports.

Classical circumstances of asphyxia-related death, known from forensic medicine textbooks, became enriched in the last years by new scenarios – they are not familiar to the emergency medicine physicians or even many crime scene investigators, and because of this it is easy to overlook them. The presence in the victim surroundings of a gas cigarette lighter or balloons and helium containers rarely draw attention to the possibility of asphyxia-related death. It is worth mentioning that both the propane-butane and helium have different theoretically possible mechanisms of action: those chemically inactive gases not only can replace oxygen rich air, especially when applied with the head put into a plastic bag or under a mini-tent, but also can cause asphyxia because of reflex constriction of the vocal cords secondary to the contact with inhaled gas that is very cold because of rapid expansion after being released from a high-pressure container. It is worth mentioning that 'getting high' by inhaling chemically non-reactive gasses from high-pressure containers seems to get more and more popular among youths these days, as it is inexpensive compared with narcotic substances and not drawing much attention. Not only can propane-butane from gas cigarette lighters or gas containers be used as a means of abuse, but also 'pressurized air' intended for the use in cleaning electronic equipment, being in fact, in most cases, a mix of organic gasses. Even in case of proper medical treatment started early on in asphyxia victims, prognosis tends to be poor because of rapid critical ischemia of the brain. Initial success in cardiopulmonary resuscitation often does not augur well, as the victim may die after several days spent in the intensive care unit; gender and age of the victim being irrelevant in this respect.

5 Conclusions

In case of violent asphyxia it is crucial to immediately undertake actions needed to save the central nervous system from imminent hypoxia – not only by removing the noose from the neck and suction of fluids from the mouth and bronchial tree, but also by rapid ventilation with pure oxygen when circumstances suggest that inhalation of a chemically inactive gas could be the cause of asphyxia. Even immediate medical help aimed at reverting hypoxia of the central nervous system followed by intensive medical treatment in the intensive care unit do not guarantee positive therapeutic outcome.

Violent asphyxia is a quite commonly established cause of death in the practice of forensic pathologists – among all the autopsies performed in 2010 every sixth was of asphyxia

victim. The most common type of deceased was an alcohol intoxicated male dead because of hanging.

Seasonal changes in the incidence of deaths by drowning have been confirmed to exist by the study results. These changes logically correspond with the weather conditions most inviting to participate in water sports and recreation. The present study confirmed that alcohol abuse is a factor strongly relating to the death by drowning.

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

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Respiratory and Systemic Infections in Children with Severe Aplastic Anemia on Immunosuppressive Therapy

57

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Abstract

In the present study we investigated the occurrence of systemic and respiratory infections in a cohort of 123 children with severe acquired aplastic anemia (SAA) on immunosuppressive therapy (IST). We recorded 101 episodes of infection in 77 patients (62.6 %). Pneumonia was among the most frequently observed clinical forms of infection (17 cases – 16.8 %). In the entire group, 23 children died, mostly in the course of fatal sepsis (15/23) and in 3 cases because of pneumonia complications. All patients were treated with horse (h-ATG) or rabbit antithymocyte globulin (r-ATG) supplemented with cyclosporine and corticosteroids. The crude incidence rate for serious infections in h-ATG group and r-ATG group was comparable. The relative risk of infectious complications was lower in patients treated with granulocyte colony stimulating factors (G-CSF) by 36 % (RR 0.64; $p < 0.0001$). The analysis confirmed that respiratory tract and disseminated infections comprise a very serious clinical problem and are the leading cause of death of SAA children. Active surveillance and the analysis of associated risk factors are required to detect opportunistic infections in this group of patients.

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DOI 10.1007/978-94-007-6627-3_57,

Keywords
Aplastic anemia • Children • Respiratory infections • Immunosupressive therapy

1 Introduction

Acquired aplastic anemia is a bone marrow failure characterized by a reduction in hematopoietic stem and progenitor cells, empty bone marrow, and pancytopenia (Young 2002). In children with severe acquired aplastic anemia (SAA), hematopoietic stem cell transplantation (HSCT) from a human leukocyte antigen (HLA)-identical sibling donor is the therapy of choice (Scheinberg and Young 2012). Combined immunosuppressive therapy (IST) which consists of antithymocyte globulin (ATG) and cyclosporine A (CSA) is an effective and safe gold standard first-line therapy for patients lacking a family matched donor (Scheinberg et al. 2008).

Persistent neutropenia is the dominant risk factor for the development of bacterial and invasive fungal infection in SAA (Valdez et al. 2009). Corticosteroids, routinely administered as serum sickness prophylaxis associated with ATG, add to the net effect of ATG + CSA in producing profound T-cell depletion and dysfunction. Children with SAA on immunosuppressive therapy are highly predisposed to infection with all types of pathogens: bacteria, viruses, fungi, and parasites, including opportunistic pathogens. The available data provide some limited evidence that the risk of infection caused by DNA viruses, including varicella zoster or herpes simplex virus (HSV), cytomegalovirus (CMV), and Epstein-Barr virus (EBV), is increased in patients during vulnerable period of impaired T-cell response (Valdez et al. 2011). Although it is demonstrated that pulmonary infections contribute to the mortality of children with severe hematological disorders, few prospective studies have been published to date on the incidence, epidemiology, and clinical impact of viral respiratory tract infections in immunecompromised children with SAA. The aim of the present study was to evaluate infectious complications, most notably respiratory infections, in children with SAA during immunosuppressive therapy.

2 Methods

2.1 Patients

The study was approved by a local Ethics Committee. The nature of the study was explained to the patients and their parents. The informed consent form was reviewed and signed by the patients or their parents/guardians. A cohort of patients studied included 123 children with SAA treated between 1993 and 2011 in 11 pediatric oncology and hematology centers in Poland belonging to the Pediatric Hematology Group. The characteristics of the patients is presented in Table 57.1.

All patients fulfilled the criteria for SAA, while 41 of them had a diagnosis of very severe SAA (vSAA). The following criteria were used to diagnose SAA: marrow cellularity <25 % of normal for the age or 25–30 % with <30 % reserve of hematopoietic cells and the presence of two out of the following three abnormalities: absolute neutrophil count (ANC) $<0.5 \times 10^9$/L, reticulocyte count $<20.0 \times 10^9$/L, and platelet count 20.0×10^9/L (Camitta et al. 1976). The very severe form was diagnosed if ANC was $<0.2 \times 10^9$/L. A diagnosis of congenital bone marrow aplasia or paroxysmal nocturnal hemoglobinuria (PNH) was excluded in all patients. PNH was excluded on the basis of flow cytometry analysis of the expression of CD55 and CD59 antigens on neutrophils and erythrocytes. Fanconi anemia was excluded on the basis of chromosome fragility test (diepoxybutane (DEB)) or mitomycin assay. None of the patients had a HLA-matched, related bone marrow donor.

Table 57.1 Characteristics of patients

Number of patients	123
Age (years) at the moment of IST initiation, range (mean)	10.5 ± 4.6
	(0.5–17.5)
Gender (boys/girls)	73 (59.4 %)
	50 (40.6 %)
Etiologic factors, number of patients (%)	
Idiopathic	57 (46.3 %)
Toxic	43 (35.0 %)
Hepatitis	23 (18.7 %)
Severity of aplastic anemia	
SAA	82 (66.7 %)
Very SAA	41 (33.3 %)

IST immunosuppressive therapy

2.2 Protocol of Therapy

Immunosuppressive therapy (IST) was given to all patients according to the European Blood and Marrow Transplant (EBMT) Severe Plastic Working Party protocol (Gluckman et al. 2002). Horse ATG (h-ATG) was used in 60 patients (48.8 %). It was administered intravenously (12–18 h infusion) in a dose of 15 mg/kg/day for 5 days together with oral prednisone in a dose of 2 mg/kg on days 1–11, 1 mg/kg on days 12–18, 0.5 mg/kg on days 19–25, and cyclosporine A (CSA) in a dose of 5 mg/kg/day orally in divided doses started on day 1 and continued for at least 6 months (till 24 months). CSA level was monitored and the dose was adjusted to maintain trough serum levels of 150–250 μg/ml.

Rabbit ATG (r-ATG) was used in 63 patients (51.2 %). It was administered intravenously (12–18 h infusion) at a dose of 3.75 mg/kg/day for 5 days together with oral prednisone and CSA administered as in above outlined. CSA level was monitored as above.

Granulocyte colony stimulating factors (G-CSF) and granulocyto-macrophage colony stimulating factor (GM-CSF) were given to 50 patients in a dose of 5 μg/kg s.c. or i.v. starting from day 1 till ANC increased to $>1.5 \times 10^9$/L or up to 28 days in the absence of the ANV increase.

For all immunosuppressed children, standardized precautions, such as special body care and disinfection measures, had to be followed by the patients themselves and the visiting family members.

2.3 Diagnosis of Infections

The infection rate was analyzed during the first 180 days of immunosuppressive therapy. Medical history was recorded and physical examination was performed according to routine clinical practice. The infections were recognized on the basis of clinical symptoms and the results of diagnostic imaging. Respiratory symptoms such as coughing accompanied by crackles or wheezing, with or without concomitant fever, were interpreted as respiratory tract infection. An infection was regarded as severe in the presence of symptoms such as tachypnea, hypoxemia, and respiratory insufficiency requiring oxygen supplementation or mechanical ventilation. Additionally, microbiological, serological (presence of specific antibodies or antigens) and molecular (presence of viral genetic material by PCR) tests were performed to find the etiological factor of the infection. The respiratory tract specimens were directly aspirated into a sterile tube and transferred to the laboratory for immediate microbiological or molecular analysis of the sample. Fungal infections were recognized according European Organization for Research

and Treatment of Cancer/Mycoses Study Group criteria (De Pauw et al. 2008).

The patients did not obtain any prophylaxis against bacterial or viral infections except the first 2 weeks of acyclovir treatment.

2.4 Statistical Analysis

The number of infections and pneumonia ratio that occurred in patients treated with h-ATG and r-ATG groups were expressed as relative risks (RR). In addition, respective RR values were calculated for the specific groups of patients (G-CSF and control groups). RR equals 1 when no increase of risk is observed. Any higher or lower value was interpreted as an increase or decrease of relative risk, respectively. Statistical analysis was performed using commercial STATA 11.0 software.

3 Results

3.1 Etiology and Clinical Presentation of Infection

In the entire group of 123 patients, 101 episodes of infection in 77 children (62.6 %) were recorded during the first 180 days of immunosuppressive therapy. The main manifestations of infection included: fever of unknown origin (FUO) in 25 (24.8 %) of cases, stomatitis in 20 (19.8 %), septicemia in 19 (18.8 %), pneumonia in 17 (16.8 %), urinary tract infection in 7 (6.9 %), enteritis in 5 (4.9 %), and other in 8 (7.9 %) of cases. Table 57.2 presents a summary of clinical data.

Stomatitis was due mainly to fungal infections diagnosed on the basis of a characteristic clinical picture. The presence of *Candida albicans* in oral swab was confirmed in 6/20 (30 %) cases. Systemic infections were mainly caused by Gram-negative bacteria 17/19 (89.5 %). Only were 2 cases of sepsis caused by Gram-positive bacteria (*Streptococcus mittis* and *Streptococcus epidermidis*). Another two cases were caused by the Gram-negative *Strenotrophomonas maltophila*; both patients died. Pneumonia was the fourth most common manifestation of infection observed in the study group. The etiology of pneumonia was confirmed in 10 out of the 17 cases (58.8 %) (Table 57.2). In 7 cases, pneumonia was diagnosed on the basis of clinical and radiological symptoms and was successfully treated with empirically with antibiotics.

In two patients, CMV infection was confirmed by PCR for viral DNA. In another two cases, *Aspergillus fumigatus* infection was detected by positive reaction for galactomannan, whereas *Cryptococcus neoformans* infection was recognized on the basis of the presence of the specific antigen in the serum of one child. *Pneumocystis jirovecii* infection was recognized in yet another two patients using a PCR method. Three children with pneumonia further developed sepsis caused by Gram negative bacteria. In theses cases the etiological factor was recognized by microbiological examination: *Klebsiella pneumoniae* was found in two and *Enterobacter cloacae* in one patient. *Enterobacter cloacae* pneumonia was complicated by pleural emphysema, which was formed despite neutropenia. All three children with pneumonia complicated by sepsis died. In contrast, all patients with pneumonia caused by CMV or fungi improved after the introduction of specific therapy.

3.2 Comparison of Infection Rate in Patients Assigned to Horse or Rabbit ATG Alone, or Supplemented with G-CSF

A total of 123 consecutive patients were assigned to receive horse ATG (60 patients) or rabbit ATG (63 patients). There were no significant differences in demographic or clinical characteristics between the two groups. In the h-ATG group, infections were noted in 36 out of the 60 children (60 %). In the group r-ATG, 41 out of the 63 children (65.1 %) developed infections during the first 6 months of therapy. The relative risk (RR) of any infectious complications was lower by 14 % in the h-ATG group compared

Table 57.2 Etiology and localization of documented infection in children with SAA

Diagnosis	Clinical picture	Etiology	Number of episodes
Confirmation: microbiological, serological, or molecular	Bacteremia	*Strenotrophomonas maltophila*	2
		E. coli	5
		Streptococcus mittis	1
		Staphylococcus epidermidids	1
		K. pneumoniae	5
		Enterobacter cloacae	5
	Urinary infection	*E. coli*	4
		P. aeruginosa	2
		K. pneumoniae ESBL (−)	1
	Enteritis	*Enterobacter cloacae ESBL* (+)	1
		Enterococus faecium VRE	1
		Enterococus faecium HLAR	1
		K. pneumoniae ESBL (+)	2
	Pneumonia	*CMV*	2
		Aspergillus spp.	2
		Cryptococcus neoformans	1
		Pneumocystis jirovecii	1
		K. pneumoniae	2
		Enterobacter cloacae	1
	Stomatitis	*Candida albicans*	6
Clinical confirmation	FUO		25
	Stomatitis		14
	Pneumonia		7
	Other:		
	Sinusitis		4
	Varicella		2
	HSV		2

SAA severe acquired aplastic anemia, *FUO* fever of unknown origin

with the r-ATG group; the difference was statistically insignificant (RR 0.86; $p = 0.42$). The crude incidence rate ratio comparing serious infections in the h-ATG and r-ATG groups was comparable. Pneumonia was recorded in 10 (16.7 %) patients in the h-ATG group and in 7 (11.1 %) patients in the r-ATG group. The relative risk of pneumonia was lower by 24 % in the r-ATG group compared with the h-ATG group; the difference was statistically insignificant (RR 0.76; $p = 0.31$).

We analyzed the effects of G-CSF or GM-CSF therapy on the incidence of infections. In the group receiving G-GSF, infections were observed in 45 out of the 87 children (51.7 %). In the group untreated with either G-CSF or GM-CSF, the episodes of infections were observed in 32 of 36 children (88.9 %). The relative risk of infectious complications in G-CSF treated patients was lower by 37 %; the difference was here statistically significant (RR 0.64; $p < 0.0001$). In children receiving G-GSF, 11 cases of pneumonia (12.6 %) were recorded. In the patients who did not receive growth factors, pneumonia was diagnosed in 6 out of the 36 patients (20 %). The relative risk of pneumonia

was reduced by 10 % by administration of growth factors, but the difference was statistically insignificant (RR 0.90; p = 0.556).

From the entire group of 123 patients, 23 children died. Fifteen out of the 23 fatal cases (65.2 %) resulted from systemic infection, and 3 of these were due to pneumonia followed by sepsis caused by Gram (−) bacteria.

4 Discussion

Prompt diagnosis of infection in SAA is essential for early introduction of specific therapy. We highlight our experience using modern bacteriological and molecular methods to diagnose bacterial, viral and fungal respiratory and systemic infections in these patients. Studies on infectious complications in patients with SAA are scarce, particularly in children (Quarello et al. 2012; Valdez et al. 2009, 2011; Torres et al. 2003; Dearden et al. 1998). According to the published data about 26–100 % patients with SAA develop severe infections (Torres et al. 2003). Particularly, infections occur during the first months after diagnosis of SAA. According to Quarello et al. (2012), 50 % of all reported infections occur up to 90 days after SAA diagnosis. The results of a multicenter randomized study of Tichelli et al. (2011) confirmed these findings. A retrospective study of Quarello et al. (2012) have shown 111 episodes of infection in 42 (53.8 %) out of the 78 patients with SAA. In 51 (45.9 %) episodes fever of unknown origin (FUO) was diagnosed, while in the remaining 60 (54.1 %) episodes an infection was documented.

4.1 Type of Infection

In a group of 123 patients with SAA of the present study, 101 cases of infectious complications in 77 (62.6 %) children were observed during the first 180 days of immunosuppressive therapy. This is consistent with the observations of other authors (Quarello et al. 2012; Tichelli et al. 2011). FOU was observed in 25 (24.8 %) cases, but in the remaining 86 (85.1 %) cases the infections were documented. Stomatitis in patients with SAA is quite common (Quarello et al. 2012; Brennan et al. 2001). Although it is considered a rather mild infection, its consequences (pain, food, and fluid intake limitation) can contribute to the development of serious conditions. The etiology of stomatitis is usually fungal, mainly *Candida albicans* and in patients with deep neutropenia and immunological disorders it may cause serious complications (Valdez et al. 2009). Stomatitis was observed frequently in our group (19.8 %) and in 30 % of cases candidiasis was confirmed by microbiological examination.

Systemic bacterial infections are grave complications of neutropenia during immunosuppressive therapy (Valdez et al. 2011; Weinberger et al. 1992). According to several reports, the majority of SAA patients suffer from infections caused by Gram-positive bacteria, followed by Gram negative bacteria, and fungi (Valdez et al. 2011; Torres et al. 2003; Weinberger et al. 1992). Torres et al. (2003) confirmed *Staphylococcus coagulas* -negative sepsis related to central intravenous catheter in 13 out of the 40 cases. In the present study we noted only two episodes of sepsis caused by Gram-positive bacteria. Recently published data show that infections caused by Gram-negative bacteria are infrequent now, as opposed to the 1960s and 1970s when Gram-negative bacteria caused the majority of infections in patients with SAA (Weinberger et al. 1992; Chuansumrit et al. 1990). Our present observations were not consistent with these findings, as 17/19 (89.5 %) episodes of systemic infections had been caused by Gram negative bacteria. The two fatal cases were caused by *Stenotrophomonas maltophilia,* an uncommon pathogen in humans. *S. maltophilia* is an aerobic gram-negative bacillus that is found in aquatic environment. It is an organism of low virulence which frequently colonizes fluids used in hospital settings (for example intravenous fluids). *S. maltophilia* usually must bypass normal host defenses to cause human infection and is quite common in patients with SAA, with a high mortality rate of about 40 % (Valdez et al. 2009; Torres et al. 2003).

Respiratory tract infections, particularly pneumonia, in children with SAA often lead to death (Rudan et al. 2011). Detection of an infectious agent is possible in approximately 12–20 % of cases. In other cases, the diagnosis is based mainly on clinical symptoms and imaging studies (X-ray and chest computer tomography) and treatment is empirical (Rudan et al. 2011). The most common respiratory pathogens in SAA patients include fungal pathogens, especially *Aspergillus* and *Zygomycetes* (Valdez et al. 2009; Torres et al. 2003). Invasive aspergillosis normally occurs only in severely immune-compromised patients and has a high mortality rate (25–90 %) (Urbaniak et al. 2011; Weinberger et al. 1992). Invasive disease is the most commonly seen in the lungs, but dissemination of *Aspergillus* to other tissues, including central nervous system, bone, kidney, and skin has been reported. Pulmonary infection was the fourth most common complication in our patients (16.8 % of all infectious episodes). In 3 cases, fungal etiology was confirmed (2 cases of *Aspergillus* infection and 1 case of cryptococcosis). All three patients were successfully treated with antifungals. *Pneumocystis jirovecii* infection is now rarely seen in immunocompromized patients as specific prophylaxis is widely used (Agrawal et al. 2011). We found only one case of pneumonia caused by this pathogen. SAA patients are predisposed to viral infections, especially of *Herpesviridae* etiology, which is associated with impaired T-cell function (Valdez et al. 2009). CMV, EBV, and HSV infections are often recurrent. CMV and EBV infections are fairly common in children with SAA during IST. Subclinical reactivation of the self-restraint virus does not require treatment in the majority of cases (Scheinberg et al. 2007). Some scientists argue that CMV pneumonia often has a fatal course in SAA children before introduction of antiviral therapy (Winston et al. 1988). In our study we found two cases of CMV pneumonia; both were successfully treated with gancyclovir.

4.2 Correlation Between Administration of Globulins and G-CSF

Antithymocyte globulin and cyclosporine are often the first line therapy for severe acquired aplastic anemia, since most patients lack a compatible sibling donor. The majority of the published data concerned horse formulation of the polyclonal antibody. In the past decade, rabbit ATG plus cyclosporine have gained attention because of its activity in relapsed and refractory SAA. In some centers, rabbit ATG has been used as the first-line therapy, and in others rabbit ATG is the only formulation currently available. In humans, rabbit ATG administration induces much deeper and more prolonged lymphopenia in comparison with the horse formulation. Moreover, patterns of viral reactivation have been shown to differ between these two agents (Scheinberg et al. 2007). Our study was originally designed to detect a difference in the prevalence of infection between the two groups. However, we did not find any difference in terms of infection rate, depending on the type of globulin used. Systemic bacterial and fungal infections in patients with SAA have a poor prognosis (Young 2002). In our material, there were 23 fatal cases, including 15 cases due to severe systemic infection. All of our patients had deep chronic neutropenia when fatal infection developed. Administration of granulocyte growth factors shortens the period of neutropenia, and thereby reduces the number of infections (Gluckman et al. 2002). This observation is consistent with our findings. A multicenter randomized EBMT study on severe acquired aplastic anemia treated with immunosuppressants showed a significant reduction of infectious complications and a shorter hospital stay with the use of G-CSF, compared with patients who did not receive G-CSF (24 % versus 82 %) (Tichelli et al. 2011). On the other hand, Torres et al. (2003) observed that 82 % of patients with

SAA had severe infections despite the use of G-GSF or GM-CSF. Kojima et al. (2002) showed no differences in the infection rate between the groups of children with SAA receiving or not G-CSF.

Our study confirms that infectious complications are a very serious problem in children with SAA treated with IST. Systemic infection, stomatitis, and pneumonia are the most commonly reported sites of infection. Active surveillance and the analysis of associated risk factors, is required to detect opportunistic infections in patients with SAA. An understanding of these clinical issues can help clinicians to assess the likelihood of certain etiology, formulate an effective diagnostic strategy, and initiate appropriate therapy.

Acknowledgements Polish Pediatric Hematology Group (PPHG): Jerzy Kowalczyk[1], Walentyna Balwierz[2], Ewa Zaleska-Czepko[2], Alicja Chybicka[3], Krzysztof Szmyd[3], Tomasz Szczepanski[4], Halina Bubala[4], Mariusz Wysocki[5], Andrzej Kurylak[5], Jacek Wachowiak[6], Dawid Szprecht[6], Wojciech Młynarski[7], Monika Bulas[7], Maryna Krawczuk-Rybak[8], Elżbieta Leszczynska[8], Tomasz Urasinski[9], Jaroslaw Peregud-Pogorzelski[9], Anna Balcerska [10], Barbara Kaczorowska-Hac[10] (Lublin[1], Kraków[2], Wrocław[3], Zabrze[4], Bydgoszcz[5], Poznań[6], Łódź[7], Białystok[8], Szczecin[9], Gdańsk[10]).

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

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M. Pokorski (ed.), *Neurobiology of Respiration*, Advances in Experimental Medicine and Biology 788,
DOI 10.1007/978-94-007-6627-3,