

Advances in Experimental Medicine and Biology 955
Neuroscience and Respiration

Mieczyslaw Pokorski *Editor*

Pathobiology of Pulmonary Disorders

Advances in Experimental Medicine and Biology

Neuroscience and Respiration

Volume 955

Editorial Board

Irun R. Cohen, The Weizmann Institute of Science, Rehovot, Israel

N.S. Abel Lajtha, Kline Institute for Psychiatric Research, Orangeburg, NY, USA

John D. Lambris, University of Pennsylvania, Philadelphia, PA, USA

Rodolfo Paoletti, University of Milan, Milan, Italy

Subseries Editor

Mieczyslaw Pokorski

More information about this series at <http://www.springer.com/series/13457>

Mieczysław Pokorski
Editor

Pathobiology of Pulmonary Disorders

 Springer

Editor

Mieczyslaw Pokorski
Public Higher Medical Professional School in Opole
Institute of Nursing
Opole, Poland

ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-3-319-49294-0 ISBN 978-3-319-49295-7 (eBook)
DOI 10.1007/978-3-319-49295-7

Library of Congress Control Number: 2017934334

© Springer International Publishing Switzerland 2017

This work is subject to copyright. All rights are reserved by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed.

The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.

The publisher, the authors and the editors are safe to assume that the advice and information in this book are believed to be true and accurate at the date of publication. Neither the publisher nor the authors or the editors give a warranty, express or implied, with respect to the material contained herein or for any errors or omissions that may have been made. The publisher remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

The book series *Neuroscience and Respiration* presents contributions by expert researchers and clinicians in the multidisciplinary areas of medical research and clinical practice. Particular attention is focused on pulmonary disorders as the respiratory tract is upfront at the first line of defense of the organism against pathogens and environmental or other sources of toxic or disease causing effects. The articles provide timely overviews of contentious issues or recent advances in the diagnosis, classification, and treatment of the entire range of diseases and disorders, both acute and chronic. The texts are thought as a merger of basic and clinical research dealing with biomedicine at both molecular and functional levels, and the interactive relationship between respiration and other neurobiological systems such as cardiovascular function, immunogenicity, endocrinology and humoral regulation, or the mind-to-body connection. The authors focus on the modern diagnostic techniques and the leading-edge therapeutic concepts, methodologies, and innovative treatments. The action and pharmacology of existing drugs and the development and evaluation of new agents are the heady area of research. Practical, data-driven options to manage patients will be considered. New research is presented regarding older drugs, performed from a modern perspective or from a different pharmacotherapeutic angle. The introduction of new drugs and treatment approaches in both adults and children also is discussed.

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

Neuromolecular and carcinogenetic aspects relating to gene polymorphism and epigenesis, involving both heritable changes in the nucleotide sequence and functionally relevant changes to the genome that do not involve a change in the nucleotide sequence, leading to disorders will also be tackled. Clinical advances stemming from molecular and biochemical research are but possible if the research findings are translated into diagnostic tools,

therapeutic procedures, and education, effectively reaching physicians and patients. All that cannot be achieved without a multidisciplinary, collaborative, bench-to-bedside approach involving both researchers and clinicians. The role of science in shaping medical knowledge and transforming it into practical care is undeniable.

Concerning the respiratory disorders, their societal and economic burden has been on the rise worldwide leading to disabilities and shortening of life span. COPD alone causes more than three million deaths globally each year. Concerted efforts are required to improve this situation, and part of those efforts are gaining insights into the underlying mechanisms of disease and staying abreast with the latest developments in diagnosis and treatment regimens. It is hoped that the articles published in this series will assume a leading position as a source of information on interdisciplinary medical research advancements, addressing the needs of medical professionals and allied health care workers, and also and will become a source of reference and inspiration for future research ideas.

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

Warsaw, Poland

Mieczyslaw Pokorski

Contents

Evaluation of Genetic Diversity of <i>Candida spp.</i> and <i>Klebsiella spp.</i> Isolated from the Denture Plaque of COPD Patients	1
D. Przybyłowska, K. Piskorska, M. Gołaś, M. Sikora, E. Swoboda-Kopeć, J. Kostrzewa-Janicka, and E. Mierzwińska-Nastalska	
Particulate Matter in the Air of the Underground Chamber Complex of the Wieliczka Salt Mine Health Resort	9
Wioletta Rogula-Kozłowska, Magdalena Kostrzon, Patrycja Rogula-Kopiec, and Artur J. Badyda	
Comparison of Thymic Stromal Lymphopoietin Concentration in Various Human Biospecimens from Asthma and COPD Patients Measured with Two Different ELISA Kits . . .	19
Katarzyna Górka, Patrycja Nejman-Gryz, Magdalena Paplińska-Goryca, Małgorzata Proboszcz, and Rafał Krenke	
Prevalence of <i>Neisseria meningitidis</i> Carriage with Identification of Serogroups and Genogroups in Professional Soldiers	29
K. Korzeniewski, M. Konior, M. Kiedrowska, E. Wódka, E. Zwolińska, and A. Skoczyńska	
Bacteriological Assessment of Pneumonia Caused by Gram-Negative Bacteria in Patients Hospitalized in Intensive Care Unit	39
A. Guzek, Krzysztof Korzeniewski, D. Tomaszewski, Z. Rybicki, and E. Zwolińska	
Whooping Cough in Adults: A Series of Severe Cases	47
K. Zycinska, M. Cieplak, M. Chmielewska, A. Nitsch-Osuch, A. Klaczko, M. Hadzik-Błaszczak, Z. Kur, and K. A. Wardyn	
Limited Clinical Significance of Dimeric Form of Pyruvate Kinase as a Diagnostic and Prognostic Biomarker in Non-small Cell Lung Cancer	51
Adam Rzechonek, Aleksandra Kaminska, Piotr Mamczur, Arkadiusz Drapiewski, and Władysław Budzyski	

Clostridium Difficile* Infection Due to Pneumonia*Treatment: Mortality Risk Models 59**

M. Chmielewska, K. Zycinska, B. Lenartowicz,

M. Hadzik-Błaszczuk, M. Cieplak, Z. Kur, and K.A. Wardyn

Predictors of Progression in IgA Nephropathy in Childhood 65

M. Mizerska-Wasiak, J. Małyk, A. Turczyn, K. Cichoń-Kawa,

A. Rybi-Szumińska, A. Wasilewska, B. Bieniaś, M. Zajączkowska,

M. Mikłaszewska, J. Pietrzyk, U. Demkow, M. Roszkowska-Blaim,
and M. Pańczyk-Tomaszewska**Index 75**

Evaluation of Genetic Diversity of *Candida* spp. and *Klebsiella* spp. Isolated from the Denture Plaque of COPD Patients

D. Przybyłowska, K. Piskorska, M. Gołaś, M. Sikora,
E. Swoboda-Kopeć, J. Kostrzewa-Janicka,
and E. Mierzwińska-Nastalska

Abstract

Yeast-like fungi and gram-negative bacilli are the most frequent potential pathogens of the respiratory tract isolated from the denture plaque of patients with chronic obstructive pulmonary disease (COPD). Dominant species among yeast-like fungi are *Candida albicans* and *Candida tropicalis*. Significant frequency is also exhibited by *Klebsiella pneumoniae* and *Klebsiella oxytoca*. The purpose of this study was to analyze genetic diversity of the strains of *C. albicans*, *C. tropicalis*, and *Klebsiella spp.* present in patients in stable phases of COPD. The analysis was conducted by the random amplified polymorphic DNA (RAPD) method on clinical strains isolated from patients with COPD and control patients in overall good health. Forty one strains of *Candida albicans*, 12 of *Candida tropicalis*, as well as 9 strains of *K. pneumoniae* and 7 of *K. oxytoca* were scrutinized. The dominant species in clinical material from COPD patients was *Candida albicans* with a substantial degree of variations of genetic profiles. On the basis of affinity analysis, 19 genetic types were identified within this strain. An analysis of the banding patterns among *C. tropicalis* strains indicated the existence of 6 genetic types. A considerable diversity of genetic profiles among *Klebsiella spp.* also was established. The genotype diversity of *Klebsiella spp.* strains may indicate the endogenic character of the majority of infections, regardless of the therapy applied for the underlying condition.

D. Przybyłowska (✉), J. Kostrzewa-Janicka, and
E. Mierzwińska-Nastalska
Department of Prosthodontics, Warsaw Medical
University, 59 Nowogrodzka Street, 02-005 Warsaw,
Poland
e-mail: dorota.przybylowska@gmail.com

K. Piskorska, M. Gołaś, and E. Swoboda-Kopeć
Department of Medical Microbiology, Warsaw Medical
University, Warsaw, Poland
M. Sikora
Department of Dental Microbiology, Warsaw Medical
University, Warsaw, Poland

Keywords

Candida spp.• COPD• Denture plaque• Genotyping• *Klebsiella spp.*
• Respiratory tract

1 Introduction

Over the past decade there has been an increased interest in the link between respiratory diseases and oral infection. Bacterial colonization may contribute directly to airway inflammation. Its products have been shown to stimulate mucin secretion and accelerated decline in lung function, and increased likelihood of death from pulmonary causes (King et al. 2013; Paju and Scannapieco 2007). Patients at the highest risk of respiratory infections, like pneumonia and bronchitis, are institutionalized patients or medically compromised patients who are unable to perform self-oral care (Scannapieco 2006). Patients with chronic obstructive pulmonary disease (COPD) are at high risk of bacterial and fungal infections, especially *Candida spp.*

Denture wearing is a promoting factor for developing mucosal infections in COPD patients. The microporous and rough surface of an acrylic denture provides conducive conditions for the accumulation of denture plaque caused through the adherence of microorganisms and debris. Denture biofilm is a mix of fungal and bacterial biofilm where complex interactions occur between *C. albicans* and other oral microorganisms. Denture stomatitis is an inflammatory process that mainly involves the palatal mucosa in oral cavity when covered by complete or partial removable dentures. This condition may affect from 15 % of generally healthy participants to more than 70 % of COPD denture wearers and is commonly complicated by a *Candida* infection secondary to long-standing occlusion of the oral mucosa by a denture. COPD patients are burdened with a high risk of bacterial and fungal infections. Previous studies indicate that multiple pathogenic bacteria species like *Klebsiella spp.* have been identified in the denture plaque of COPD patients. There are also

more cases where denture stomatitis complicated by mucosal fungi infections is acknowledged to COPD patients than to control subjects, which is explained as related to inhaled chemotherapy and home oxygen therapy through the mouth (Przybyłowska et al. 2015). Although *Candida spp.* does not normally cause disease, in cases where immune defenses are compromised or the regular microflora balance is disrupted, *Candida* transforms itself into an opportunistic pathogen, the leading cause of invasive fungal disease. Despite the high frequency of commensal carriage of *C. albicans* and its prominence as a major fungal pathogen, little has been known about its genetic homogeneity, evolution, and persistence during commensalism or parasitism (Heo et al. 2011). The opportunistic pathogen *Klebsiella spp.* usually originates in immunocompromised individuals who are hospitalized and carry underlying illnesses such as chronic pulmonary obstruction (Vogel et al. 1999). The principal reservoirs of *Klebsiella spp.* are the gastrointestinal tract and the hands of hospital personnel.

The aim of the study is to estimate the genetic diversity of *C. albicans*, *C. tropicalis* and *K. pneumoniae*, *K. oxytoca* isolated from the denture plaque of COPD patients and healthy users of dental prostheses, by means of the random amplified polymorphic DNA (RAPD) technique.

2 Methods

The study was approved by the Internal Review Board of Warsaw Medical University in Poland. The material consisted of 41 *C. albicans*, 12 *C. tropicalis*, 9 *K. pneumoniae*, and 7 *K. oxytoca* clinical strains cultured at the Warsaw Medical University Clinical Hospital

between 2013 and 2015. Every strain came from a single patient. Clinical samples were cultured out of the denture plaque obtained by direct swabs from COPD patients in stable phases of disease and from generally healthy participants. Yeast strain isolates were cultured on Sabouraud medium with chloramphenicol at 30 °C for 48 h, then inoculated through CHROMagar *Candida* medium (Graso Biotech; Starogard Gdanski, Poland), and incubated at 35 °C for 72 h to specify colony phenotype. *Candida* spp. strains were identified with an automated ID32C test (BioMerieux, Marcy l'Etoile, France) in accordance with the producer's guidelines. Bacterial strains were inoculated in Mueller Hinton agar. Bacterial diagnostics was performed through the application of the below specified procedures. The samples were submitted for growth directly on plates of 5 % sheep blood agar and Mueller Hinton agar, and were incubated under aerobic conditions at 37 °C for 24 h. The *K. oxytoca* and *K. pneumoniae* isolates were identified by the API 20 E test (BioMerieux, Marcy l'Etoile, France) and maintained as frozen stocks at –70 °C.

Genomic DNA isolation from *Candida* spp. and *Klebsiella* spp. was performed with EurX Genomic DNA Purification Kit, according to the producer's guidelines (EurX, Gdansk, Poland). Purified DNA elution was performed with Tris-EDTA buffer and stored for further analysis at –20 °C.

In the present study we used RAPD-PCR technique due to its relatively cost-effectiveness and matching the resolving power of electrophoretic karyotyping. The reaction was carried out in a DNA Engine thermal cycler (MJ Research/BioRad, Hercules, CA) under the following protocol: 95 °C for 5 min, then 45 cycles of 95 °C for 1 min, 36 °C for 1 min, and 72 °C for 2 min in the respective groups of isolates. The products of the reaction were separated by electrophoresis in 2 % agarose gel with the addition of ethidium bromide. For typing *C. albicans* and *C. tropicalis* isolates, primer OPR 15: 5' GGACAACGAG3' (Gene Tools, Syngene; Cambridge, UK) was applied, and for typing *K. oxytoca* and *K. pneumoniae* – primer ERIC-2: 5' AAGTAAGTGACTGGGGTGAGC- 3'

(Amersham Pharmacia Biotech; Piscataway, NJ) was applied. Similarity coefficients were calculated based on the absence or presence of bands (Fig. 1). Dendrograms of similarity coefficients were generated using a binary code according to Gene Tools (Cambridge, UK). In the present study, the interpretation of results was as follows: for 1.0 similarity coefficient, the isolates were designated as genetically indistinguishable; for 0.99–0.80 coefficients, they were considered closely related (highly similar but not identical, could be considered the same strain); for 0.79–0.50 coefficients they were considered possibly related; and for less than 0.50 coefficient, they were considered unrelated (Rodrigues et al. 2008; Soll 2000). The cut-off value of similarity coefficient ≥ 0.80 was arbitrarily used as the threshold for clustering of similar genotypes.

3 Results

A total of 40 *C. albicans* isolates were obtained from the cohort of COPD patients and one isolate from among the healthy participants using dental prostheses. Denture swabs were collected from eight patients which were then treated with bronchodilators (B), 11 with a combination of bronchodilators and inhaled steroids (B + S), 11 with bronchodilators and home oxygen therapy (B + O), and 10 patients with a combination of all medicines (B + S + O). On the basis of RAPD profiles, from among the 40 strains of *C. albicans* 19 genotypes, from A to T, were established within the overall yeast population, which included from 2 to 5 isolates. We observed four genotypic unique strains (C, F, H, and K) as shown in the dendrogram (Fig. 1). Seven genotypes were derived at the threshold similarity coefficient of 0.80. Only did Cluster B with the coefficient > 0.80 include two strains coming from the patients treated with bronchodilators (B), suggesting highly related isolates. The remaining genotypes with the coefficient < 0.80 were unrelated (Figs. 1 and 2).

A total of 12 *C. tropicalis* isolates were scrutinized. We grouped them into 6 main RAPD clusters. Two of them were derived at

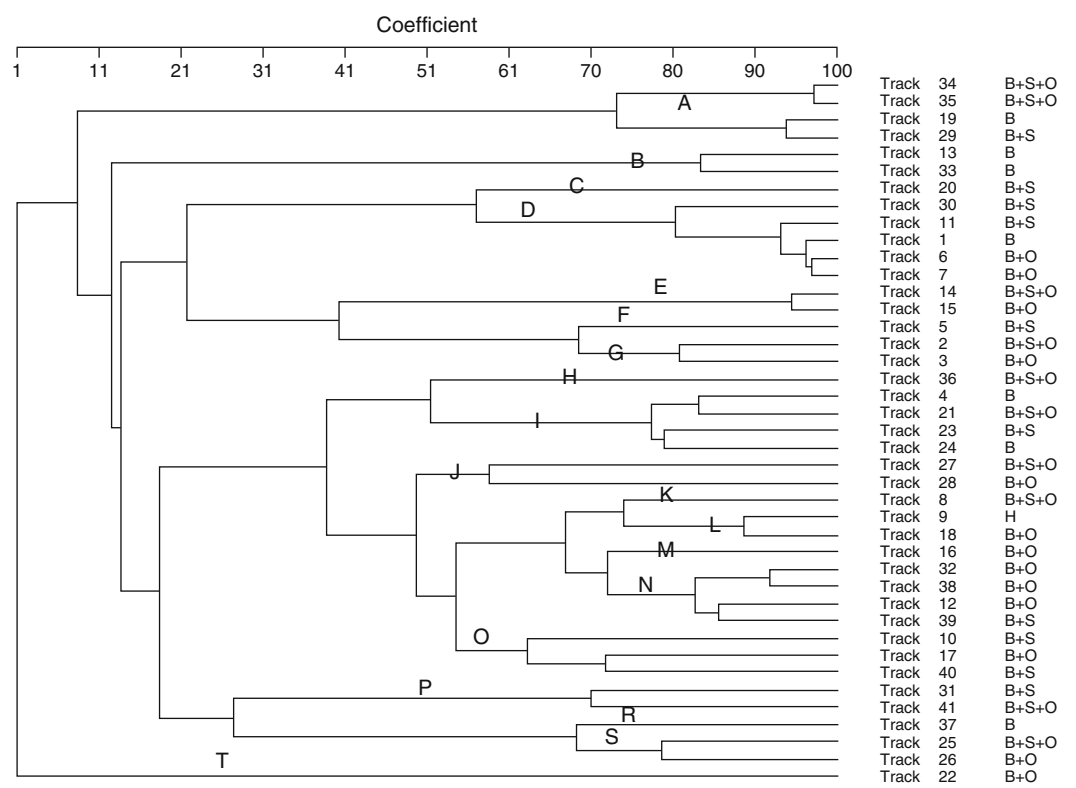


Fig. 1 Hierarchical clustering (UPGMA dendrogram) generated for 40 isolates of *Candida albicans* from denture plaque of patients with COPD on the basis of RAPD-PCR profiles. Participants were divided into following groups: *B* threated with bronchodilators, *B + S* bronchodilators with inhaled corticosteroids, *B + O* bronchodilators with home oxygen therapy, and *B + S + O* combinations of all medicines, *H* healthy denture user, *A-T* represent major genotypes

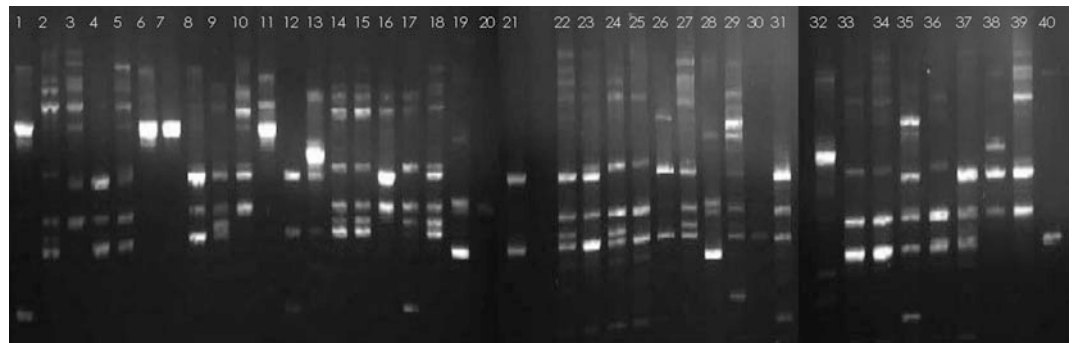


Fig. 2 RAPD profiles of *Candida albicans* isolates amplified by primer ERIC-1

the threshold similarity coefficient of 0.80 (*A* and *E*). Cluster *A* included four isolates from the patients treated with different types of pharmacological therapy and from one healthy participant. Only did Cluster *E* include isolates from the patients treated with bronchodilators and steroids (*B + S*). We observed also one cluster, *F*, which included isolates from the

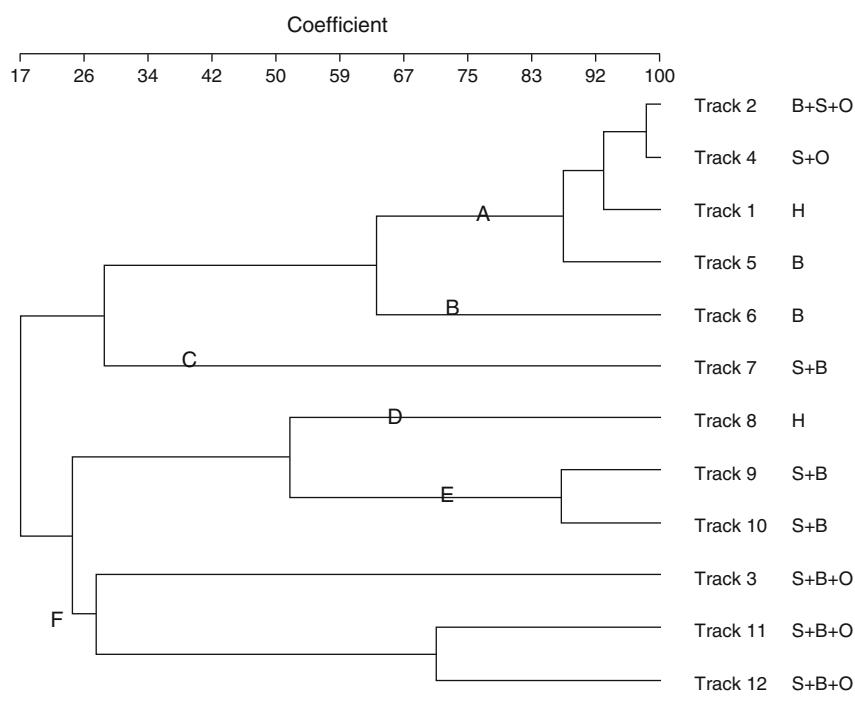


Fig. 3 Hierarchical clustering (UPGMA dendrogram) generated from the similarity coefficient which shows interindividual similarity. Twelve isolates of *Candida*

tropicalis were isolated from users of dental prostheses with COPD and from two healthy participants

patients who were treated with a combination of bronchodilators, steroids, and home oxygen therapy (B + S + O), but with a low coefficient of relatedness (Fig. 3).

We examined nine *K. pneumoniae* isolates. All of them were grouped into 5 main clusters (from A to E), and two unique strains (B and E). One cluster, A, had a similarity coefficient of 0.80. Only was one genotype observed in a patient who was treated with bronchodilators and steroids (B + S) (Fig. 4). There was no material obtained from healthy participants in this group.

On the basis of seven *K. oxytoca* RAPD fingerprint profiles we defined 4 major clusters (A to D) (Fig. 5). Surprisingly, only one was derived at a similarity level of nearly 0.80 and consisted of two isolates that presented different patterns (Cluster A). Cluster B consisted of three isolates, which included strains from healthy participants and from one patient with a combination of COPD therapy, but it could not be considered related.

4 Discussion

The identification and classification of *Candida* in oral candidosis are important for diagnosis, investigations of epidemiology and pathogenesis, as well as for the treatment of the main disease, especially in immunocompromised patients. The presence of the opportunistic pathogen *C. albicans* in oral cavity in COPD patients is considered an important factor in the development of mucosa membrane inflammation under the removable acrylic denture. Our previous study has revealed that poor denture hygiene may be responsible for high levels of *Candida* spp. and Gram-negative bacteria. There were also more cases where denture stomatitis was complicated by infection of mucosal fungi in COPD patients than in control healthy subjects (Przybyłowska et al. 2015).

Random amplified polymorphic DNA is one of the most frequently used methods for epidemiological investigations of fungal and bacterial

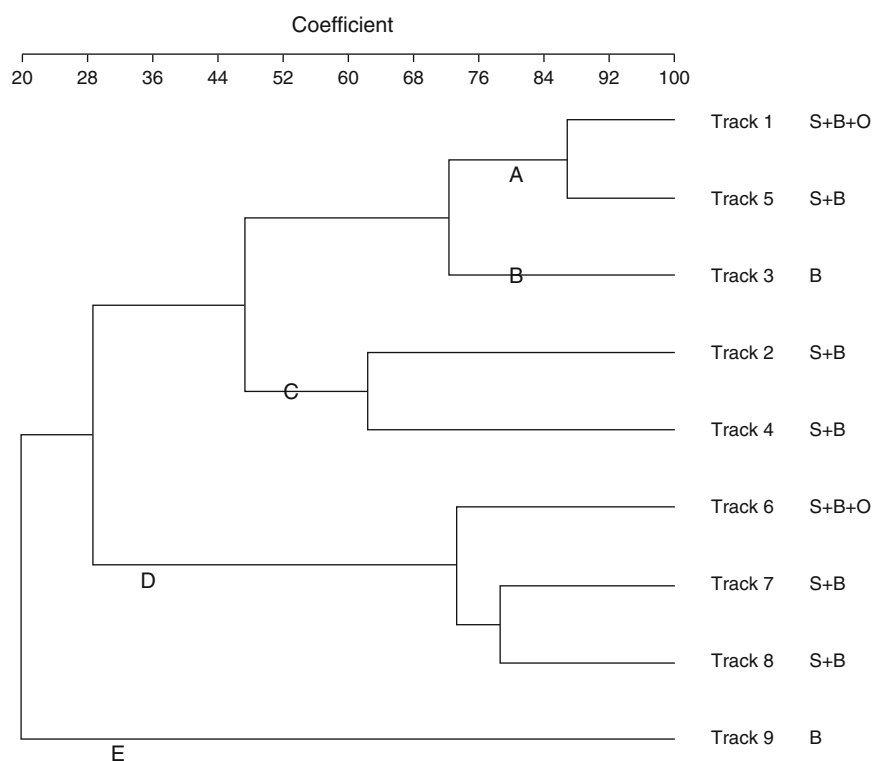


Fig. 4 Hierarchical clustering (UPGMA dendrogram) analysis of *Klebsiella pneumoniae* isolates based on RAPD typing

infections. RAPD technique is easier to perform than other methods, it is rapid, cost-effective and useful for the typing of a large number of strains (Noumi et al. 2009). PCR-based methods have certain limitations such as the DNA concentration, amplification parameters, and the type of thermal cycler (MacPherson et al. 1993). Moreover, the lack of reproducibility is a problem with using short and nonspecific primers under low-stringency conditions. The results of Ashayeri-Panah et al. (2012) have shown that pulsed-field gel electrophoresis (PFGE) and optimized random amplified polymorphic DNA (RAPD) techniques are comparable and equally valuable for the typing of both *K. pneumoniae* and *C. albicans* nosocomial isolates (Costa et al. 2008). Jain et al. (2001) have recommended this technique to differentiate *C. albicans* strains resistant to fluconazole isolated from AIDS patients. Noumi et al. (2009) have compared

two karyotyping techniques, RAPD and contour-clamped homogenous electric fields (CHEF) electrophoresis, to identify clonal-related isolates from oral mucosa membrane and vaginal sites. They conclude that the genotype of each isolate and genotypic difference between *C. albicans* and *C. glabrata* isolates are patient specific and not associated with the site of infection, geographic origin, or date of isolation. Previous research have assessed the genetic similarity of *C. albicans* isolates associated with dental prosthesis in diabetic hemodialyzed patients and healthy individuals. RAPD profiles with a high similarity coefficient greater than 0.90, demonstrate that these isolates are highly related. The plausibility arises that *C. albicans* strains may adapt to the similar oral conditions in diverse individuals and may reproduce in a clonal way (Pires-Gonçalves et al. 2007). Song et al. (2006) have revealed

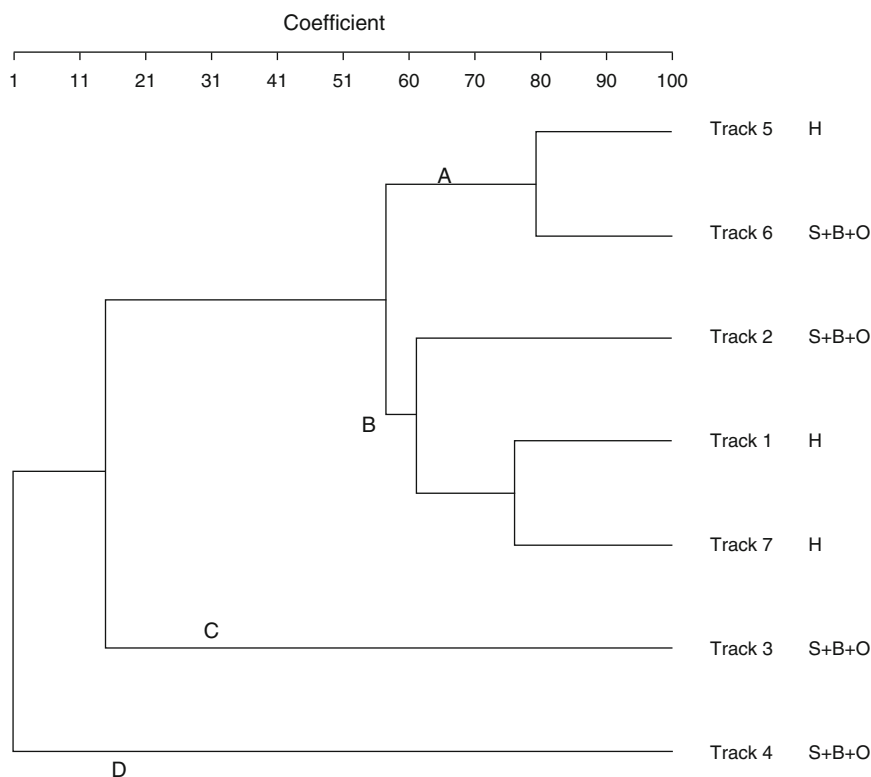


Fig. 5 Hierarchical clustering (UPGMA dendrogram) of *Klebsiella oxytoca* strains based on RAPD typing using primer ERIC-2. Isolates have been taken from denture

users with COPD treated with a combination therapy (B + S + O) and from healthy participants (H)

that the yeasts of these commonest forms of oral candidosis are genetically more diverse than yeasts from orally healthy subjects.

The present study demonstrates a substantial differentiation of *C. albicans* and *C. tropicalis*, as well as *Klebsiella spp.* That suggests the lack of endemic occurrence of strains and indicates that the vast majority of invasive fungal infections have an endogenic character, which is consistent with other reports in the literature. Overall, we found a high degree of heterogeneity among the fungal and bacterial isolates from COPD patients and healthy participants. There was no correlation between the kind pharmacotherapy employed and RAPD fingerprints. da Costa et al. (2012) have also demonstrated genetic diversity of *C. albicans* and *C. tropicalis* strains isolated from oral swabs taken from patients.

5 Conclusions

This study addressed the genotypic distribution of pathogenic organisms isolated from COPD patients wearing removable dentures. The examination covered *Candida spp.* and *Klebsiella spp.* genotypes and was conducted with the use of RAPD-PCR method. The findings demonstrate a large genetic variability among the strains isolated from the denture plaque of COPD patients as well as from healthy subjects. The patients, irrespective of treatment combination, had highly similar, but not identical strains, in the denture plaques, which suggests that these strains could adapt to the oral environmental condition in a kind of evolutionary way.

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

References

- Ashayeri-Panah M, Eftekhari F, Feizabadi MM (2012) Development of an optimized random amplified polymorphic DNA protocol for fingerprinting of *Klebsiella pneumoniae*. *Lett Appl Microbiol* 54(4):272–279
- Costa F, Manaia CM, Figueiral MH, Pinto E (2008) Genotypic analysis of *Candida albicans* isolates obtained from removable prosthesis wearers. *Lett Appl Microbiol* 46:445–449
- da Costa KR, Ferreira JC, Lavrador MA, Baruffi MD, Candido RC (2012) Virulence attributes and genetic variability of oral *Candida albicans* and *Candida tropicalis* isolates. *Mycoses* 55(3):e97–e105
- Heo SM, Sung RS, Scannapieco FA, Haase EM (2011) Genetic relationships between *Candida albicans* strains isolated from dental plaque, trachea, and bronchoalveolar lavage fluid from mechanically ventilated intensive care unit patients. *J Oral Microbiol* 3:6362. doi:10.3402/jom.v3i0.6362
- Jain P, Khan ZK, Bhattacharya E, Ranade SA (2001) Variation in random amplified polymorphic DNA (RAPD) profiles specific to fluconazole-resistant and -sensitive strains of *Candida albicans*. *Diagn Microbiol Infect Dis* 41(3):113–119
- King PT, MacDonald M, Bardin PG (2013) Bacteria in COPD; their potential role and treatment. *Transl Respir Med* 1:13
- MacPherson JM, Eckstein PE, Scoles GJ, Gajadhar AA (1993) Variability of the random amplified polymorphic DNA assay among thermal cyclers, and effects of primer and DNA concentration. *Mol Cell Probes* 7:293–299
- Noumi E, Snoussi M, Saghrouni F, Ben Said M, Del Castillo L, Valentin E, Bakhrouf A (2009) Molecular typing of clinical *Candida* strains using random amplified polymorphic DNA and contour-clamped homogenous electric fields electrophoresis. *J Appl Microbiol* 107(6):1991–2000
- Paju S, Scannapieco F (2007) Oral biofilms, periodontitis, and pulmonary infections. *Oral Dis* 13(6):508–512
- Pires-Gonçalves RH, Miranda ET, Baeza LC, Matsumoto MT, Zaia JE, Mendes-Giannini MJ (2007) Genetic relatedness of commensal strains of *Candida albicans* carried in the oral cavity of patients' dental prosthesis users in Brazil. *Mycopathologia* 164(6):255–263
- Przybyłowska D, Mierzwińska-Nastalska E, Rubinsztajn R, Chazan R, Rolski D, Swoboda-Kopec E (2015) Influence of denture plaque biofilm on oral mucosal membrane in patients with chronic obstructive pulmonary disease. *Adv Exp Med Biol* 839:25–30
- Rodrigues MVP, Fusco-Almeida AM, Nogueira NGP, Bertoni BW, Torres SCZ, Pietro RCLR (2008) Evaluation of the spreading of isolated bacteria from dental consulting-room using rapid technique. *Lat Am J Pharm* 27(6):805–811
- Scannapieco F (2006) Pneumonia in nonambulatory patients: the role of oral bacteria and oral hygiene. *J Am Dent Assoc* 137(10):21S–25S
- Soll DR (2000) The ins and outs of DNA fingerprinting the infectious fungi. *Clin Microbiol Rev* 13(2):332–370
- Song X, Sun J, Støre G, Eribe ER, Hansen BF, Olsen I (2006) Genotypic relatedness of yeasts in thrush and denture stomatitis. *Oral Microbiol Immunol* 21(5):301–308
- Vogel L, Jones G, Triep S, Koek A, Dijkshoorn L (1999) RAPD typing of *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Serratia marcescens* and *Pseudomonas aeruginosa* isolates using standardized reagents. *Clin Microbiol Infect* 5(5):270–276

Particulate Matter in the Air of the Underground Chamber Complex of the Wieliczka Salt Mine Health Resort

Wioletta Rogula-Kozłowska, Magdalena Kostrzon,
Patrycja Rogula-Kopiec, and Artur J. Badyda

Abstract

This study evaluates the mass concentration and chemical composition of particulate matter (PM), collected in the chamber complex of the underground health resort located in the Wieliczka Salt Mine in southern Poland. Physical and chemical properties of PM were examined from the standpoint of their possible connection with therapeutic effects of the subterranean air in the mine. We found that in three underground spots we measured the average concentration of PM did not exceed $30 \mu\text{g}/\text{m}^3$. Chemical composition of PM was dominated by sodium chloride, making up 88 % of its mass, on average. It was shown that the underground ambient concentration of PM and its chemical composition depended mostly on the nature of the rock material present in the ventilation tunnel of the health resort, filtering the incoming air. The presence and effect of external sources of PM, including patients' activity, also had an impact on the underground PM concentration.

Keywords

Chemical composition • Dust • Health effects • Particulate matter • Salt aerosol • Speleotherapy • Subterraneotherapy

W. Rogula-Kozłowska and P. Rogula-Kopiec
Institute of Environmental Engineering, Polish Academy
of Sciences, Zabrze, Poland

M. Kostrzon (✉)
Wieliczka Salt Mine Health Resort, 1 Park Kingi Street,
Bldg. I, 32-020 Wieliczka, Poland
e-mail: magdalena.kostrzon@kopalnia.pl

A.J. Badyda
Faculty of Environmental Engineering, Warsaw
University of Technology, Warsaw, Poland

1 Introduction

Subterraneotherapy or speleotherapy is a specific treatment approach belonging to the climatotherapeutical methods. The basic therapeutic factor is a set of climatic characteristics formed in the underground chambers or caves created during excavations, frequently being mining for salt (Ponikowska and Ferson 2008; Beamon et al. 2001). The value of subterraneotherapy is based

on a synergistic influence of a complex of physical, chemical, and biological stimuli on the human organism (Skulimowski 1964). The air composition of subterranean salt chambers promotes treatment of chronic respiratory ailments, notably of allergic origin. Studies demonstrate that the key factors responsible for the therapeutic effects of subterranean atmosphere are the air purity, the high concentration of sodium chloride, and the repeatable stimulating effect of a microclimate, evoked by physical properties of the surrounding environment including temperature, humidity, and air pressure (Obtułowicz 2013; Horvath 1986).

An aerosol is a colloidal system of solid or liquid particles dispersed in the gaseous phase. The composition of the mine aerosol includes substances both desirable due to their health-promoting qualities, particularly NaCl, and undesirable in this regard organic and inorganic compounds considered air pollutants, mostly the material carried underground with the air coming from the external environment or connected with the presence of people and equipment in the mine. Humidity and temperature conditions in the subterranean environment cause that well-soluble salts, such as NaCl, occur mostly in the dissociated state in mine aerosol, which enhances their inhalational absorption in the respiratory tract. Salts become an inherent component of the underground ambient air as a result of erosion of internal walls occurring during movement of humid air through the underground tunnels and salt chambers. The concentration of these compounds may reach several dozen milligrams per one cubic meter of air.

Therapeutic potential of the underground ambient air is based on the microbiological content and on the quantity and chemical composition of the air aerosol, understood as a mass of solid or liquid phases separated on a filter medium from one cubic meter of the air (Frączek et al. 2013; Obtułowicz et al. 2013). Particulate matter (PM) defines the pollutant fraction of an aerosol. The amount of PM in the air is standardized by the global (WHO 2013; European Directive 2008/50/EC; European Directive 2004/107/EC) and national regulations

(Decree of the Minister of Work and Social Policy 2002). The impact of PM on the human organism depends on the particles size, their capability to migrate *via* the respiratory system, and the content of toxic components (Pope and Dockery 2006; Englert 2004). Concerning the health effects, concentration of solid particles in the inhaled air counts the most. When PM concentration is too high, even in case of a favorable chemical composition, it may adversely affect persons suffering from bronchial asthma or chronic obstructive pulmonary disease, possibly by enhancing bronchial responsiveness, cough, and the like (Kim et al. 2015).

As the ambient air in the mine is *de facto* atmospheric air sucked from the surface, the quantitative and qualitative composition of PM in mine excavation areas are determined by the properties of solid particles originating from the atmospheric air. However, the path length of the air flowing into the underground chambers, and the type of rocks forming the ventilation tunnel may be of no less importance, particularly in case of salt mines, in purification of the atmospheric air entering the mine.

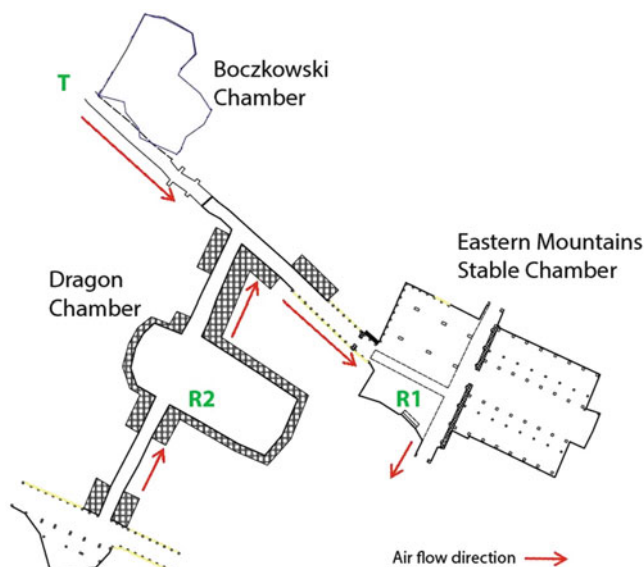
The Wieliczka Salt Mine Health Resort offers therapies profiled for pneumologic and allergologic ailments. The salt chambers are located at level III of the mine, 135 m deep. The movement of air in the mine is enforced by ventilation fans installed in the outtake shafts. The path of airflow through the underground excavation areas is approximately 700–800 m long. On average, about 180 m³/min of air moves through the chambers of the health resort.

The goal of this study was to evaluate the chemical composition and concentration of PM collected in the chamber complex of the Wieliczka Salt Mine Health Resort to get insight into the potential factors shaping beneficial effects of subterraneotherapy.

2 Methods

The study was approved by an institutional Review Board for Research. The protocol consisted of the sampling of PM by aspiration

Fig. 1 Locations of measurement points in the underground salt chamber complex: Eastern Mountains Stable Chamber (R1), Dragon Chamber (R2), and Boczkowski chamber (T) of the Wieliczka Salt Mine Health Resort



from the underground ambient air and of the determination of chemical composition of the material sampled. The investigation was performed in the period of July 7–20, 2015.

Three measurement points were selected for the investigation: two chambers of the Health Resort: Eastern Mountains Stable Chamber (R1; rehabilitation room) and Dragon Chamber (R2; gym hall), and the air supply tunnel to the chamber complex (T) at the Boczkowski Chamber (Fig. 1). Samples of total particulate matter (TPM; airborne dust particles with the aerodynamic diameter of 1 nm to 100 μm) were simultaneously collected at each measurement point. Additionally, a sample of respirable particulate matter (PM_{10} ; airborne dust particles with the aerodynamic diameter of 1 nm to 4 μm) was collected in the tunnel at the Boczkowski Chamber.

Air testing required a collection of app. 9–10 m^3 of air for a single measurement. Assuming the average adult breathing frequency of 15 times *per min* and air intake of 0.5 dm^3 *per breath*, the volume of the test air was approximately equivalent to that inhaled by a patient during three eight-hour sessions in the Health Resort (app. 3 m^3 *per session*). A single measurement lasted for app. 72 h, with the air flow set at

2–2.5 dm^3/min to ensure the optimal measurement quality. The measurements were repeated thrice in each room, covering app. a 10-day time. In this way, tests took into account the influence of air quality, environmental conditions, and patients' activity on chemical composition of PM. As there are no regulations for the methodology of studies on PM in the underground ambient air of a mine or a health resort, the measurements were carried out according to the Polish standards determining the measurement conditions in workplaces (PN-91/Z-04030/05 1991). The PM sample collection was performed using Gilian sampling pumps (Sensidyne, St. Petersburg, FL). Samples were collected on Whatman quartz filters (QMA, $\phi 25$ mm) (GE Medical Systems Polska, Life Sciences, Warsaw, Poland).

The mass of a PM sample was determined by filter weighing on a microscale (resolution of 1 μg) (Radwag; Radom, Poland) before and after the exposure. Before weighing, chemical composition on the filter was conditioned in the weighing room for 48 h under constant conditions (humidity of $45 \pm 5\%$ and air temperature of $20 \pm 2^\circ\text{C}$).

After the collection of aerosol samples and gravimetric analysis, a fraction of each collected

Table 1 Concentration of particulate matter (PM) in three underground chambers of the Health Resort in three consecutive measurement periods

	No. of measurement	Date of measurement	PM	Mass concentration ($\mu\text{g}/\text{m}^3$)
R1 Eastern Mountains Stable Chamber	1	2015/06/7-10	TPM	33.9
	2	2015/06/10-13		42.4
	3	2015/06/13-16		23.3
R2 Dragon Chamber	1	2015/06/7-10		27.1
	2	2015/06/10-13		37.6
	3	2015/06/13-16		24.8
T Tunnel in Boczkowski Chamber	1	2015/06/7-10		15.5
	2	2015/06/10-13		17.0
	3	2015/06/13-16		24.7
	1	2015/06/7-10	PM ₄	8.5
	2	2015/06/10-13		6.8
	3	2015/06/13-16		7.8

PM particulate matter, TPM total particulate matter, PM₄ respirable particulate matter of 1 nm to 4 μm in diameter

sample was taken for tests on organic carbon (OC) and elemental carbon (EC) contents using a thermal-optical method. The remaining part of the aerosol collected on the filter was extracted in water and the content of the following ions was determined by ion-exchange chromatography: Cl^- , NO_3^- , NO_2^- , Br^- , PO_4^{3-} , SO_4^{2-} , F^- , I^- , Na^+ , NH_4^+ , K^+ , Ca^{2+} , and Mg^{2+} . The analytical methods, conditions of extraction, and chromatographic analysis, including precision verification procedures, have been described in detail elsewhere (Rogula-Kozłowska 2016).

3 Results and Discussion

In both chambers of the Health Resort – *Eastern Mountains Stable* (R1) and *Dragon* (R2), the concentration of TPM in the air was similar and, on average, amounted to app. 30 $\mu\text{g}/\text{m}^3$ during the first 72-h measurement period. TPM in either chamber differed significantly in the consecutive measurement periods, by as much as app. 10 $\mu\text{g}/\text{m}^3$ (Table 1), indicating that averaging the fluctuating TPM over several measurement periods would lack substance. Overall, TPM concentration in the underground chambers may be considered low. For comparison, the TPM subfraction PM₁₀, i.e., airborne dust particles of 1 nm to 10 μm in diameter, exceeds 40 $\mu\text{g}/\text{m}^3$ in a school of the city of Wrocław

(Zwoździak et al. 2013) or reaches 80 $\mu\text{g}/\text{m}^3$ in the Wawel Museum in Cracow in winter months (Worobiec et al. 2010). TPM concentration is reported to go over 160 $\mu\text{g}/\text{m}^3$ in nursery schools in the city of Gliwice in the Upper Silesia region (Mainka et al. 2015).

The average TPM concentration in the supply air tunnel (T) was distinctly lower than that in the chambers, by about 10 $\mu\text{g}/\text{m}^3$ (Table 1). Less than half of TPM at the inlet of air to the underground chambers was made up by respirable PM₄ particles (average concentration of 7 $\mu\text{g}/\text{m}^3$). It is noteworthy that such concentration of respirable PM ranks as exceptionally low. In the atmospheric air of the so-called Green Lungs of Poland area (Borki Forest), which is assumed as being of highest air quality, the average yearly concentration of respirable PM reaches 15 $\mu\text{g}/\text{m}^3$ and it amounts to 30 $\mu\text{g}/\text{m}^3$ at the coastal city of Gdansk (Rogula-Kozłowska et al. 2014). In Cracow (distance from Wieliczka Salt Mine of 12 km in a straight line), average yearly concentration of respirable PM is at the level of 46–64 $\mu\text{g}/\text{m}^3$; reaching as high as 100–190 $\mu\text{g}/\text{m}^3$ in winter months (average daily concentration depending on the measurement point location). The air introduced underground from the outside is mechanically filtered out at the intake opening of the ventilation tunnel, which ensures removal of coarse solid particles. However, atmospheric PM consists of mostly fine and ultrafine particles

(Rogula-Kozłowska 2016). PM forms minute nucleation particles or very fine particles forming as a result of transformation of gaseous precursors, in various proportions depending on a current meteorological condition and chemical composition of air (Chow et al. 2015; Seinfeld and Pandis 2006). Submicrometer particles, PM_{10} , constitute 50–70 % of TPM (Rogula-Kozłowska et al. 2013). It is then clear that the exceptionally low TPM and PM_{10} concentrations in T are due mostly to air purification occurring while the air travels through the ventilation tunnel system of the mine rather than the effect of air filtering at the very inlet of the ventilation tunnel. Presumably, the main structural material of tunnel and chamber walls NaCl absorbs outside air and anthropogenic contaminants (Fig. 2).

Each of the three collected TPM and PM_{10} samples at the inlet of air to the chambers of the Health Resort (point T) mainly consists of Na^+ and Cl^- . Sulfate (SO_4^{2-}), ammonium (NH_4^+), calcium (Ca^{2+}), and phosphate (PO_4^{3-}) ions belong to lower fractions in PM. All the determined ions of water-soluble compounds (salts) of PM constitute, on average, 90 % of TPM and 88 % of PM_{10} at point T, the tunnel inlet to the Health Resort (Fig. 3). They may be either ions originating from compounds occurring in the

atmospheric air, reaching point T *via* the tunnel, or connected with the natural structural elements of the mine walls. On the other side, organic carbon and elemental carbon may be considered typical anthropogenic pollutants (Chow et al. 2015; Chow 1995). In the PM reaching the Boczkowski Chamber, minimal amounts of organic carbon (app. 2 %; Fig. 2) are present. For comparison, the fraction of organic carbon in the respirable PM reaches 20 % in the sea coastal region of Gdansk or 30–50 % in southern Poland cities of Zabrze and Katowice (Rogula-Kozłowska et al. 2014; Rogula-Kozłowska et al. 2012).

Low fractions of both undetermined components and organic carbon in PM at point T, could be due to the powerful cleansing of atmospheric air on the way through the ventilation tunnel. That however would not be true for elemental carbon whose content at point T remained comparable to the level present in the respirable PM in Polish cities (Rogula-Kozłowska et al. 2014; Rogula-Kozłowska et al. 2012). Thus, elemental carbon fraction in PM (Fig. 3) may be considered an indicator of air pollution with external solid particles at the inlet to the underground chambers of the Health Resort. The content of elemental carbon found

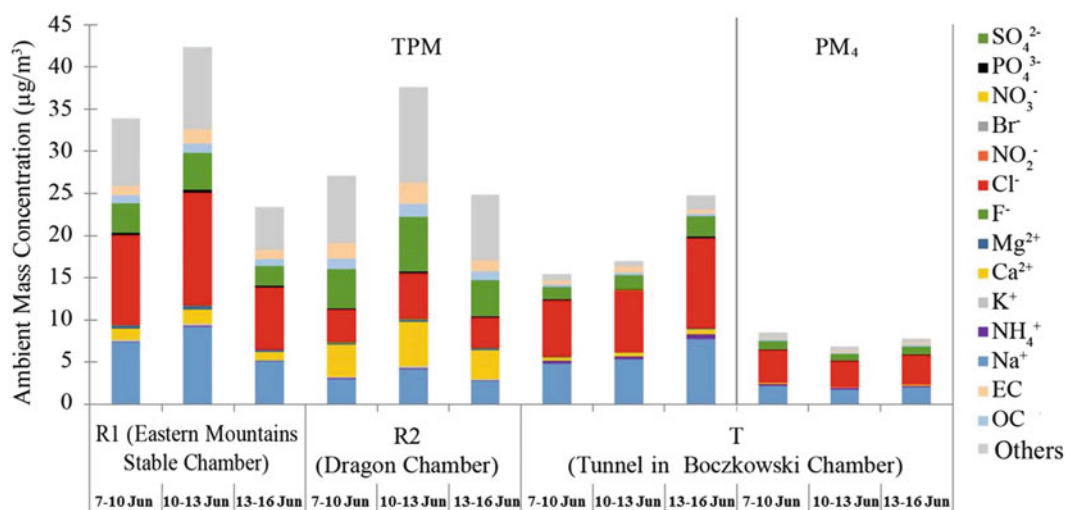


Fig. 2 Concentration of PM components in the underground chambers of the Wieliczka Salt Mine Health Resort in consecutive measurements periods. *EC* elemental carbon, *OC* organic carbon, *Others* undetermined compounds

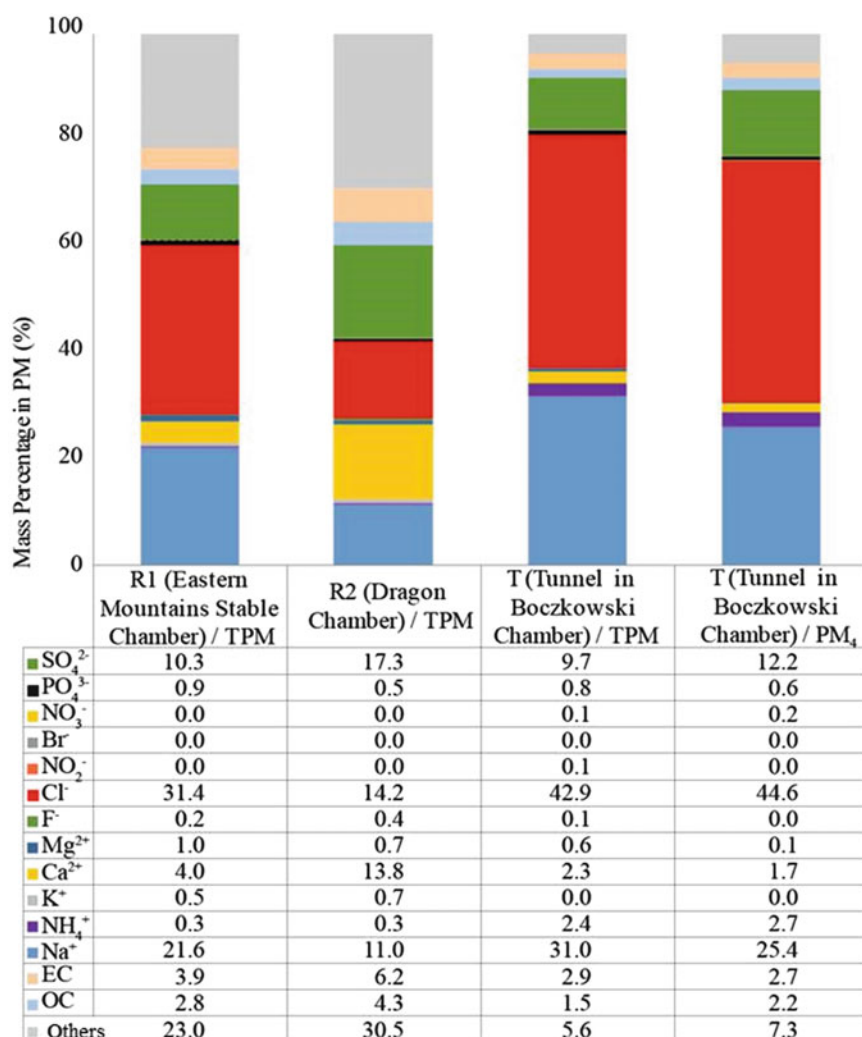


Fig. 3 Average percentage of PM components in the underground chambers of the Wieliczka Salt Mine Health Resort in consecutive measurements periods. *EC* elemental carbon, *OC* organic carbon, *Others* undetermined compounds

at point T does not pose a hazard for the respiratory function.

Ions of water-soluble compounds prevail in TPM in all the chambers (Figs. 2 and 3). The undetermined substances (marked as 'others' in the figures) as well as carbon compounds, both organic and elemental, have a higher fraction in R1 and R2 therapeutic chambers than that present in the air at point T, the tunnel inlet to the Health Resort. In all likelihood, this difference in TPM chemical composition in both therapeutic chambers in relation to the incoming air at point T is connected with the particle emission

resulting from human residence inside the Health Resort. All actions of patients and employees of the Health Resort, life processes, and the pollutant load brought in by the patients (clothing, skin, hair, food, together with pollutants settled on their surfaces), and also the use of furniture, equipment, rehabilitation devices, are bound to increase TPM in the air inside the chambers. The impact on health of environmental PM is well recognized (Lippman 2007).

Irrespective of the differences in TPM chemical composition in the three underground locations of the Health Resort, this composition

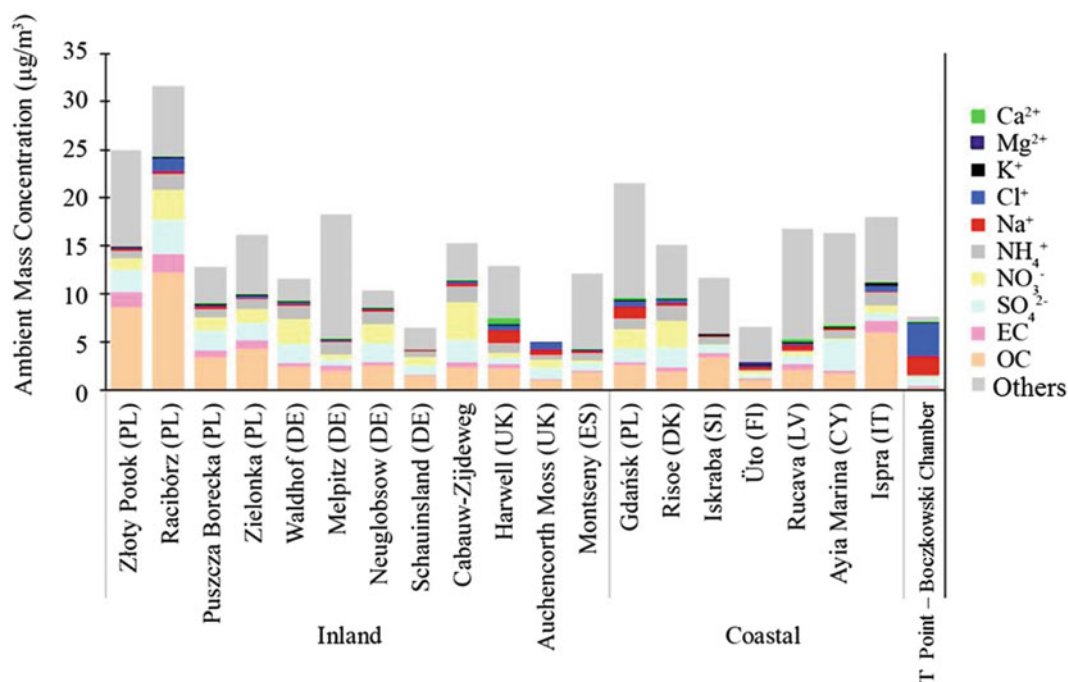


Fig. 4 Ambient content of respirable PM in various European inland and coastal locations in comparison with the underground air content at T point, i.e., the tunnel inlet of air in the Boczkowski Chamber of the Wieliczka Salt Mine Health Resort (last right-hand bar)

(Drawn up on the basis of the European monitoring of air quality data: <http://ebas.nilu.no>; <http://www.eea.europa.eu/data-and-maps/data/airbase-the-european-air-quality-database-7>, and data of Rogula-Kozłowska et al. 2014)

was entirely different from that present in the outside ambient air in that the strongly dominant ions in the underground chambers, starting from T point at the inlet to the Boczkowski Chamber, were Na^+ and Cl^- , forming a type of saline saturated air. The difference between the underground and outside air content was distinctly clear even taking into account differences between various European inland and coastal areas (Fig. 4). Inland, fractions of Na^+ and Cl^- ions in respirable PM are considered to be connected with burning fuels and biomass rather than with the possible influence of marine aerosol. In coastal locations, on the other hand, marine salt occurs both in fine- and coarse-particle forms, although its influence covers a zone limited to several hundred meters offshore (Rogula-Kozłowska et al. 2014; Rogula-Kozłowska et al. 2012; Seinfeld and Pandis 2006).

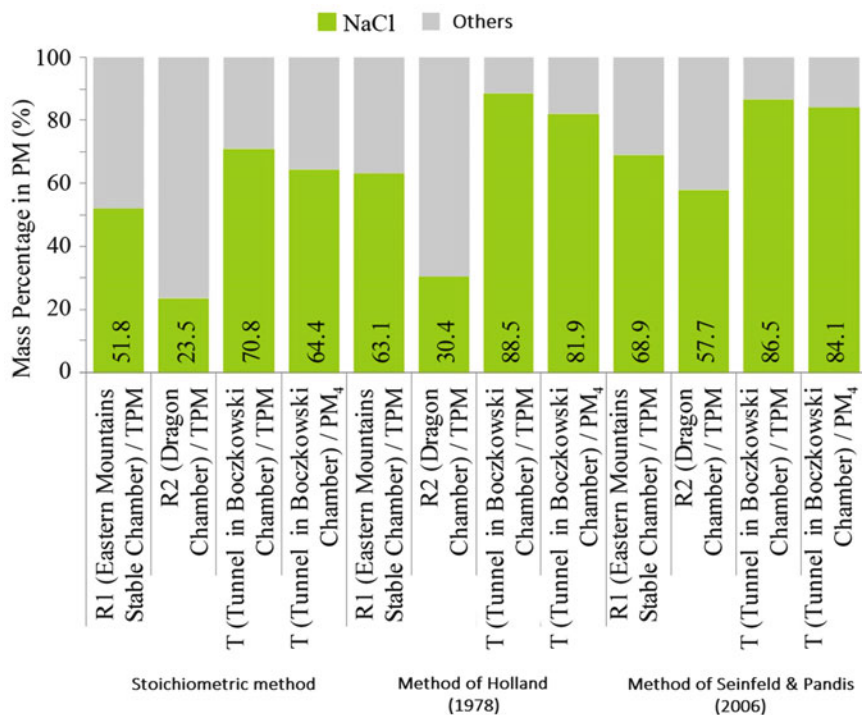
It may be presumed that a low concentration of respirable PM in the underground ambient air

and the dominant presence of sodium and chloride ions are conducive to therapy of ailments of respiratory airways. From the therapeutic standpoint, concentration of solid particles of NaCl in PM is more important than the amount of Na^+ and Cl^- ions in PM. This concentration is determinable from the amount of respective ions. The simplest method is to calculate the ratio of sodium and chloride moles, using a stoichiometric evaluation. Other methods take into account a possible underestimation of chloride concentration in the air, introducing specific coefficients (Seinfeld and Pandis 2006; Holland 1978). In the present study, determination of NaCl concentration in the underground air of the Health Resort was carried out with three different methods in each of the three measurement locations. The results of these calculations are shown in Table 2 and Fig. 5. It ought to be recognized that the calculations provide approximate results,

Table 2 Estimated NaCl concentration of particulate matter (PM) in three underground chambers of the Health Resort in three consecutive measurement periods

	No. of measurement	Date of measurement	PM	NaCl concentration ($\mu\text{g}/\text{m}^3$)		
				Stoichiometric method	Method of Holland (1978)	Method of Seinfeld and Pandis (2006)
R1 Eastern Mountains Stable Chamber	1	2015/06/7-10	TPM	17.6	21.4	20.7
	2	2015/06/10-13		21.9	26.7	25.9
	3	2015/06/13-16		12.1	14.7	14.3
R2 Dragon Chamber	1	2015/06/7-10		6.4	8.3	12.2
	2	2015/06/10-13		8.8	11.4	16.8
	3	2015/06/13-16		5.8	7.6	11.1
T Tunnel in Boczkowski Chamber	1	2015/06/7-10		11.0	13.7	12.2
	2	2015/06/10-13		12.0	15.0	13.4
	3	2015/06/13-16		17.5	21.9	19.6
	1	2015/06/7-10	PM ₄	5.5	7.0	6.4
	2	2015/06/10-13		4.4	5.6	5.1
	3	2015/06/13-16		5.0	6.4	5.8

PM particulate matter, TPM total particulate matter, PM₄ respirable particulate matter of 1 nm to 4 μm in diameter

**Fig. 5** Average estimated fractions of NaCl in particulate matter (PM) of the aerosol mass in the underground chambers of the Wieliczka Salt Mine Health Resort based on the concentration of the compound's ions

as it is assumed that given components forms a given compound as a whole, which is hardly true in nature.

4 Conclusions

The present study demonstrates that total particulate matter and, in particular, respirable particulate matter were both distinctly low in the underground chambers of the Wieliczka Salt Mine Health Resort compared with the known levels of air-borne pollutants in the ambient air that is outside in various areas, even those with assumedly better air quality such as coastal areas. The exact reason for the excellent underground air quality is not entirely clear as the outside air is pumped into the mine through the system of ventilatory tunnels. We believe, however, our results provided a rational explanation for the underground air quality, which is purification of the outside air on its way through the ventilatory tunnels due to the nature of structural material overlying the tunnels walls; the dominant component of that material is NaCl. The explanation is reinforced by the incomparable better air quality, in terms of lower PM, at the point of air leaving the ventilation tunnels, i.e., outlet to the therapeutic underground chambers, than the outside air quality. A somehow higher PM content in the therapeutic chambers that follow the route of air flow, still much lower than that in the outside air, would be due to the anthropogenic pollutants originating from human activity inside the chambers.

The concentration of NaCl in PM of the underground chambers of the Salt Mine, approaching 90 %, was several times higher than that present in various coastal areas. This high content of NaCl, along with specific composition of solid particles in the air, creates a specific microclimate and aerosanitary conditions that may explain therapeutic benefits of subterraneotherapy observed in bronchial asthma or chronic obstructive pulmonary disease (Kostrzon et al. 2015). We believe that the present study makes an important contribution to the

limited knowledge on the pulmonary effects of a subterranean microclimate.

Acknowledgements Data analysis was supported by the DEC-2013/09/N/ST10/04224 project, which was financed by the National Science Center.

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

References

- Beamon SP, Falkenbach A, Fainburg G, Linde K (2001) Speleotherapy for asthma. *Cochrane Database Syst Rev* 2:CD001741
- Chow JC (1995) Measurement methods to determine compliance with ambient air quality standards for suspended particles. *J Air Waste Manag Assoc* 45:320–382
- Chow JC, Lowenthal DH, Chen LW, Wang X, Watson JG (2015) Mass reconstruction methods for PM_{2.5}: a review. *Air Qual Atmos Health* 8(3):243–263
- Decree of the Minister of Work and Social Policy (2002) on the maximum admissible concentrations and intensities of the factors harmful to health in the workplace. *Journal of Laws of the Republic of Poland*. 2002 (217), item 1833 with subsequent amendments
- Englert N (2004) Fine particles and human health: a review of epidemiological studies. *Toxicol Lett* 149:235–242
- European Directive 2004/107/EC of the European Parliament and of the Council of 15 December 2004 relating to arsenic, cadmium, mercury, nickel and polycyclic aromatic hydrocarbons in ambient air
- European Directive 2008/50/EC of the European Parliament and of the Council of 21 May 2008 on ambient air quality and cleaner air for Europe
- Frączek K, Górny RL, Ropek D (2013) Bioaerosols of subterraneotherapy chambers at salt mine health resort. *Aerobiologia* 29(4):481–493
- Holland HD (1978) *The chemistry of the atmosphere and oceans*. Wiley-Intersciences, New York
- Horvath T (1986) Speleotherapy: a special kind of climatotherapy, its role in respiratory rehabilitation. *Int Rehabil Med* 8:90–92
- Kim KH, Kabir E, Kabir S (2015) A review on the human health impact of airborne particulate matter. *Environ Int* 74:136–143
- Kostrzon M, Czarnobilski K, Czarnobilska E (2015) The influence of pulmonary rehabilitation in the Wieliczka Salt Mine on asthma control-preliminary results. *Przegl Lek* 72:716–720
- Lippman M (ed) (2007) *Environmental toxicants: human exposures and their health effects*, 3rd edn. Wiley, New York

- Mainka A, Zajusz-Zubek E, Kaczmarek K (2015) PM_{2.5} in urban and rural nursery schools in Upper Silesia, Poland: trace elements analysis. *Int J Environ Res Public Health* 12:7990–8008
- Obtułowicz K (2013) Mechanism of therapeutic effects of subterranean therapy in the chambers of the Salt Mine Wieliczka. *Alergol Immunol* 10:26–29
- Obtułowicz K, Myszkowska D, Dyga W, Mazur M, Czarnobilska E (2013) Hypoallergic subterranean therapy in salt chambers of Wieliczka Mine of the therapy of airways and skin allergy. The role of bioaerosol. *Alergol Immunol* 10:2–3 (in Polish)
- PN-91/Z-04030/05 (1991) Air purity protection. Tests for dust. Determination of total dust in workplaces by filtration-gravimetric method. Polish Committee on Normalization, Measurements, and Quality, Warsaw (in Polish)
- Ponikowska I, Ferson D (2008) Modern spa medicine. MediPress, Warsaw, pp 191–196 (in Polish)
- Pope CA, Dockery DW (2006) Health effects of fine particulate air pollution: lines that connect. *J Air Waste Manage Assoc* 56:709–742
- Rogula-Kozłowska W (2016) Size-segregated urban particulate matter: mass closure, chemical composition, and primary and secondary matter content. *Air Qual Atmos Health* 9(5):533–550
- Rogula-Kozłowska W, Klejnowski K, Rogula-Kopiec P, Mathews B, Szopa S (2012) A study on the seasonal mass closure of ambient fine and coarse dusts in Zabrze, Poland. *Bull Environ Contam Toxicol* 88:722–729
- Rogula-Kozłowska W, Klejnowski K, Rogula-Kopiec P, Ośródka L, Krajny E, Błaszczak B, Mathews B (2014) Spatial and seasonal variability of the mass concentration and chemical composition of PM_{2.5} in Poland. *Air Qual Atmos Health* 7:41–58
- Rogula-Kozłowska W, Kozielska B, Klejnowski K (2013) Hazardous compounds in urban PM in the central part of Upper Silesia (Poland) in winter. *Arch Environ Prot* 39:53–65
- Seinfeld JH, Pandis SN (2006) Atmospheric chemistry and physics: from air pollution to climate change, 2nd edn. Wiley, New York
- Skulimowski M (1964) Treatment of asthmatics in chambers of the Salt Mine in Wieliczka. *Przegl Lek* 4–5:225–228
- WHO (2013) Health effects of particulate matter. Policy implications for countries in Eastern Europe, Caucasus and central Asia. WHO Regional Office for Europe, Copenhagen
- Worobiec A, Samek L, Krata A, Van Meel K, Krupinska B, Stefaniak AE, Karaszkiewicz P, Van Grieken R (2010) Transport and deposition of airborne pollutants in exhibition areas located in historical buildings-study in Wawel Castle Museum in Cracow, Poland. *J Cult Herit* 11:354–359
- Zwoździak A, Sówka I, Krupińska B, Zwoździak J, Nych A (2013) Infiltration or indoor sources as determinants of the elemental composition of particulate matter inside a school in Wrocław. *Poland Build Environ* 66:1–27

Comparison of Thymic Stromal Lymphopoietin Concentration in Various Human Biospecimens from Asthma and COPD Patients Measured with Two Different ELISA Kits

Katarzyna Górską, Patrycja Nejman-Gryz,
Magdalena Paplińska-Goryca, Małgorzata Proboszcz,
and Rafał Krenke

Abstract

Thymic stromal lymphopoietin (TSLP) seems a promising asthma biomarker. In earlier studies, mainly the serum concentration of TSLP was investigated. The aim of the present study was to compare the TSLP concentration measured by two different ELISA kits in the serum, induced sputum, and exhaled breath condensate in asthma, COPD, and control subjects. The study included 24 asthmatics, 36 patients with COPD, and 12 controls. TSLP concentration was measured with the use of R&D and EIAab commercial ELISA kits. The results obtained with the EIAab kit were 3 to even 45-fold higher than those measured with the R&D kit. Significant differences between the investigated groups were found only for the TSLP concentration in induced sputum. When the R&D kit was used, the highest TSLP levels in induced sputum were found in asthmatics, while the EIAab kit showed the highest TSLP levels in controls. The distribution of results in the Bland-Altman plot was typical for a proportional constant error. TSP concentration in induced sputum might be a more reliable asthma biomarker than serum TSLP. We conclude that TSLP level is highly dependent on the ELISA kit used for the measurement. Thus, judgement on TSLP results obtained with different assays might be confusing and lead to wrong conclusions.

K. Górską, P. Nejman-Gryz (✉), M. Paplińska-Goryca,
M. Proboszcz, and R. Krenke
Department of Internal Medicine, Pneumology and
Allergology, Medical University of Warsaw, 1A Banacha
Street, 02-097 Warsaw, Poland
e-mail: patrycja.nejman-gryz@wum.edu.pl

Keywords

Airway obstruction • Exhaled breath condensate • Induced sputum • Serum • TSLP

1 Introduction

Thymic stromal lymphopoietin (TSLP) is a cytokine with pleiotropic activity produced mainly by epithelial cells in response to different pro-inflammatory stimuli (Liu et al. 2007). This cytokine was first described as a growth factor for B lymphocytes identified in the conditioned medium of the murine thymic stromal cell line (Levin et al. 1999). Later, it has been shown that TSLP can be produced by variety of human cells, including epithelial cells, fibroblasts, mast cells, macrophages, and granulocytes (Sokol et al. 2008; Ying et al. 2005). The highest TSLP expression was demonstrated in the epithelial cells of airways and skin (Watson and Gauvreau 2014).

TSLP belongs to the 4-helix bundle cytokine family that includes six conserved cysteine residues (Leonard 2002). The human *TSLP* gene is located on chromosome 5q22.1 (Quentmeier et al. 2001) and its alternative splicing results in two isoforms of this protein: long and short which consist of 159 and 60 amino acids (Reche et al. 2001). The two translational products of the *TSLP* gene have different biological properties and different expression under normal conditions and during inflammation (Fornasa et al. 2015).

It has been shown that TSLP production can be induced by a variety of factors including the following pro-inflammatory cytokines: tumor necrosis factor alpha (TNF- α), interleukin (IL)-1, interferon beta (IFN- β), tumor growth factor beta (TGF- β), IL-4, IL-13, the ligands of Toll-like receptor, gut commensals, bacterial and viral infections, and allergens (Ying et al. 2015; Kato et al. 2007). In human airway epithelial cells, expression of TSLP is regulated by IL-1b and TNF- α in a nuclear factor (NF)- κ B-dependent manner (Lee and Ziegler 2007).

TSLP may affect various types of hematopoietic cells, including dendritic cells,

mast cells, natural killer T-cells, eosinophils, and basophils triggering allergic inflammation (Noti et al. 2013; Cook et al. 2012). TSLP signaling is mediated through a heterodimeric receptor complex, which is composed of the IL-7 receptor alpha-chain (IL-7R α) and TSLP receptor chain (TSLPR). TSLP receptor is a type of I transmembrane protein, which belongs to the hematopoietin receptor superfamily (Pandey et al. 2000; Park et al. 2000).

There is some evidence that TSLP plays an important role in the development of Th2-dependent immune response (Liu et al. 2007). Some studies have shown that airway TSLP mRNA expression is increased in asthma patients compared with healthy subjects, and correlates with disease severity (Liu 2006; Ying et al. 2005). Moreover, anti-TSLP antibody reduced allergen-induced airway response (Gauvreau et al. 2014). Other studies have identified the *TSLP* gene as the locus associated with susceptibility to allergic diseases which influences the serum IgE level (Hunninghake et al. 2008), eosinophil count (Gudbjartsson et al. 2009), and the initiation of allergic inflammation by dendritic cells (Ito et al. 2012). Zhou et al. (2005) have demonstrated an increased lung TSLP expression in mice with antigen-induced asthma, whereas TSLP receptor-deficient animals had significantly attenuated allergic inflammation. Some authors have reported a single-nucleotide polymorphism in the human *TSLP* gene and suggested that a difference in the regulation of TSLP expression can affect susceptibility to allergic diseases (Ferreira et al. 2014; Harada et al. 2011). On the other hand, others have reported an increased TSLP level in broncho-alveolar lavage fluid (BALF) of COPD patients compared with healthy subjects (Ying et al. 2008), but a role of TSLP in COPD is poorly known. Thus, TSLP can be considered as a

reasonable therapeutic target for the future preventive and therapeutic strategies of inflammatory airway disorders.

The above *in vitro* and *in vivo* findings lead to the hypothesis that airway TSLP expression might contribute to the control of a local airway response to inhaled allergens. If so, the measurement of TSLP in respiratory samples seems critical in further studies on its role in allergic airway diseases, including asthma. It should be noted that in the majority of previous studies mainly the serum concentration of TSLP has been measured (Watanabe et al. 2015; Lee et al. 2010). There are few data on TSLP levels in other biological materials, including BALF, bronchial mucosa, or human airway smooth muscle cells (Ying et al. 2008; Zhang et al. 2007). Compared to other cytokines involved in the allergic response, the measurement of TSLP poses problems, due mainly to the complexity of cytokine release and the lack of appropriate reagents. Our preliminary study (data unpublished) has shown significant differences in TSLP concentration depending on the ELISA kits used for the measurement. Therefore, we undertook a study aimed at the systematic comparison of TSLP concentration measured by two different commercial ELISA kits in various biospecimens obtained from the patients with asthma, COPD, and from the control subjects.

2 Methods

2.1 Study Design

The study protocol was approved by the Bioethics Committee of the Medical University of Warsaw in Poland (permit KB/186/2010). All the study participants had signed informed consent.

This was a prospective, cross-sectional study that included three well-defined groups of patients: asthmatics, patients with chronic obstructive pulmonary disease (COPD), and control subjects. Serum, exhaled breath condensate (EBC) and induced sputum (IS) were collected from study participants and these materials were used to measure TSLP concentration. In all

samples TSLP level was assessed using two different ELISA kits.

2.2 Study Participants

Twenty four patients with mild-to-moderate asthma, 36 patients with mild-to-moderate COPD, and 12 control subjects were enrolled. Asthma and COPD diagnoses and severity assessment were based on the Global Initiative for Asthma (GINA) and Global Initiative for Chronic Obstructive Lung Disease (GOLD) publications, respectively (GINA 2015; GOLD 2015). In all patients the obstructive airway disease was in stable phase. The major exclusion criteria for asthma and COPD patients were as follows: 1/disease exacerbation or respiratory infection during 6 weeks before the study onset and 2/treatment with inhaled or oral steroids within 6 weeks preceding sample collection.

All patients underwent routine clinical evaluation that included medical history, physical examination, and additional studies according to the clinical diagnosis (e.g., pulmonary function tests with bronchial obstruction reversibility assessment, skin prick tests, serum IgE, or total and differential leukocyte count in peripheral blood). Spirometry and bronchial reversibility testing (Lungtest 1000 MES, Cracow, Poland) were performed according to the recommendations of the European Respiratory Society (ERS) (Pellegrino et al. 2005). Patients in the control group had a normal spirometry and negative history of obstructive lung diseases.

2.3 Sample Collections

Serum samples were obtained by centrifugation of 10 mL of venous blood collected from the peripheral vein and were stored at -70°C .

Sputum induction was performed with sterile hypertonic saline (NaCl) at increasing concentrations (3 %, 4 %, and 5 % solutions) *via* an ultrasonic nebulizer (ULTRA-NEBTM2000, DeVilbiss, USA) in accordance with the ERS guidelines (Djukanović et al. 2002; Vignola

et al. 2002). Sputum plugs were isolated from saliva and were processed with 0.1 % solution of dithiothreitol. The obtained supernatants were stored at -70°C for TSLP measurements.

The exhaled breath condensate was collected and processed according to the American Thoracic Society/ERS (Horváth et al. 2005) using the TURBO-DECCS 09 system (Medivac; Parma, Italy) during tidal breathing for 20 min and -5°C condensation temperature, as described previously (Górská et al. 2016). The samples were portioned into 500 μL aliquots and immediately stored at -70°C for subsequent analysis. The EBC samples were not lyophilized. None of the above samples were repeatedly thawed/frozen for analysis.

2.4 TSLP Analysis

TSLP concentration was measured in all specimens of serum, IS, and EBC with two different commercially available kits:

- ELISA Kit for Human Thymic stromal lymphopoietin (EIAab WUHAN, China, Cat. No E1320h) with a detection range of 31.2–2000 pg/mL and sensitivity 5.6 pg/mL. This assay recognizes recombinant and natural human TSLP. According to the manufacturer no significant cross-reactivity or interference is observed.
- Human TSLP Quantikine ELISA Kit (R&D Systems, Cat. No DTSLP0; Minneapolis,

MN) with a detection range of 31.3–2000 pg/mL and sensitivity 9.87 pg/mL.

All procedures were performed in accordance with the manufacturer's instructions. TSLP concentrations were simultaneously measured with both ELISA kits immediately after thawing each sample.

2.5 Statistical Analysis

Data are presented as medians and interquartile ranges (IQR) or means with 95 % confidence intervals (95 %CI). To verify whether the data were normally distributed the Shapiro-Wilk test was used. The differences between continuous variables in unrelated groups were tested with the non-parametric Mann-Whitney U and Kruskal-Wallis tests. To assess the agreement between the results of measurements performed with two different ELISA kits the Bland and Altman (B&A) plot was used. Statistical significance was accepted at a p-value less than 0.05. Statistical analyses were performed using Statistica 10.0 (StatSoft Inc., Tulsa, OK) and MedCalc Statistical Software version 13.2.2 (MedCalc Software bvba, Ostend, Belgium).

3 Results

Basic demographic and clinical characteristics of patients are presented in Table 1.

Table 1 Demographic and clinical data on patients with asthma, chronic obstructive pulmonary disease (COPD), and controls

Parameter	Asthma ($n = 24$)	COPD ($n = 36$)	Controls ($n = 12$)
Male/female (n)	11/13	21/15	6/6
Age (yr)	51 (31–61)	67 (60–72)	55 (36–64)
Never-smokers/ex-smokers/current smokers (n)	21/3/0	0/20/16	7/3/2
Cigarette smoke exposure (packyears)	0 (0–0)	45 (34–60)	0 (0–20)
Positive skin prick-tests, n (%)	15 (62.5)	7 (19)	2 (17)
Pre-bronchodilator FEV ₁ (L)	2.6 (1.9–3.9)	1.6 (1.2–2.0)	2.9 (2.1–3.8)
Post-bronchodilator FEV ₁ (L)	2.7 (2.1–4.3)	1.8 (1.3–2.2)	–
Pre-bronchodilator FEV ₁ (%)	89 (77–96)	67 (54–74)	99 (91–106)
Post-bronchodilator FEV ₁ (%)	99 (81–103)	70 (64–80)	–

FEV₁, forced expiratory volume in one second; Results are medians with interquartile range (IQR), unless otherwise stated

The ultimate analysis included only those samples, which were available for the measurement of TSLP concentration using both ELISA kits. Therefore, the number of serum, induced sputum, and exhaled breath condensate samples subjected for final analysis was lower than the total number of study participants. There were 69 serum samples (24 asthma, 34 COPD, and 11 controls), 30 sputum samples (9 asthma, 17 COPD, and 4 controls), and 68 EBC samples (23 asthma, 34 COPD, and 11 controls).

3.1 TSLP Concentration in Samples from Asthmatics, COPD Patients, and Control Subjects

TSLP concentration in various samples from asthma, COPD, and controls is presented in three major columns of Table 2.

There were significant differences in sputum TSLP concentration between asthma, COPD, and control subjects. The measurements performed with R&D ELISA kit showed the highest values in asthmatics, while according to EIAab ELISA kit the highest TSLP concentrations were found in control subjects. There were no significant differences in the serum or EBC levels of TSLP in asthma, COPD, and controls. When the TSLP level was compared in the specimens in a given patient groups, the lowest TSLP was found in

EBC. The TSLP level of the same order of magnitude in the three patient conditions was found only in the serum measurements obtained with the same ELISA kit.

3.2 Comparison of TSLP Concentration Measured with Different ELISA Kits

TSLP concentration measured in the same sample using both R&D and EIAab kits differed significantly, with the only exception of IS results obtained in the asthma patients. In the remaining specimens, TSLP concentration measured with EIAab kit was 3 to even 45-fold higher than the level measured with R&D kit (Table 2).

In all serum samples, TSLP concentration was in the range of detection limit of both ELISA kits. There were no serum samples with TSLP level below the lower limit of detection. All, except one, individual serum TSLP values were higher for EIAab compared to R&D. The range of serum TSLP level obtained with EIAab was much wider (18.3–1705.0 pg/mL) than that with R&D kit (7.1–325.9 pg/mL).

In 16 (53 %) sputum samples, TSLP concentration was higher for EIAab than that for R&D. In 10 (33 %) sputum samples, TSLP concentration was below the detection limit for EIAab and in 2 (6.5 %) for R&D ELISA kit. In those

Table 2 TSLP concentration (pg/mL) in serum, induced sputum, and exhaled breath condensate of patients with asthma, chronic obstructive pulmonary disease (COPD), and controls, measured by R&D and EIAab ELISA kits

Material	ELISA kit	Asthma	COPD	Controls	p-value asthma/COPD/controls
Serum	R&D	36.5 (32.6–61.1)*	38.2 (28.3–66.7)*	65.9 (44.0–90.7)*	0.24
	EIAab	740 (560–835)	620 (238–903)	895 (625–1380)	0.12
Induced sputum	R&D	50.9 (34.2–73.2)	10.8 (0–41.5)	12.2 (7.4–19.2)*	0.02
	EIAab	50.7 (0–855)	37 (0–319)	550 (325–728)	0.05
Exhaled breath condensate	R&D	0 (0–0)*	0 (0–0)*	0 (0–0)*	1.00
	EIAab	0 (0.0–41.7)	0 (0.0–38.3)	40 (0.0–76.7)	0.28

Results are medians with interquartile range (IQR). Significant differences across TSLP levels in samples from asthma, COPD, and controls are marked by p-values in bold in the rightmost column (Kruskall-Wallis test)

*P < 0.05 for differences between TSLP levels in the same specimen measured by R&D vs. EIAab ELISA kit (Mann–Whitney U-test)

2 samples, TSLP levels were also below the detection limit of EIAab kit. TSLP range in IS was 0.0–1435.0 pg/mL for EIAab and 0.0–103.7 pg/mL for R&D kit.

The lowest TSLP concentration was found in EBC. In all 68 EBC samples, TSLP was below the detection limit of R&D ELISA kit. The respective percentage of EBC samples in which TSLP was below the detection limit of EIAab ELISA kit was 54 % (37 samples). EBC concentration of TSLP measured with EIAab kit ranged between 0.0 and 660.5 pg/mL.

The matching of TSLP measurements performed with R&D and EIAab ELISA kits in various samples is presented in the form of Bland–Altman plots in Fig. 1. There were significant differences between the results of R&D and EIAab ELISA kits with respect to all evaluated materials, i.e., serum, IS, and EBC. In virtually all samples, the difference between TSLP concentration measured by EIAab and R&D kits consisted of higher values obtained with EIAab compared with R&D kit. The mean differences in TSLP level for serum, IS, and EBC were 656.2 pg/mL, 251.7 pg/mL, and 39.2 pg/mL, respectively. The distribution of points representing individual samples was characteristic for proportional constant error: the difference between EIAab and R&D results was small in samples with low TSLP concentration and proportionally increased in samples with higher TSLP levels.

4 Discussion

The major finding of this study was that the TSLP level in the same biological sample differed considerably depending on the ELISA kit used for the measurement. Except for the sputum TSLP level in asthma and COPD, all other comparisons of TSLP levels measured with R&D *versus* EIAab ELISA kits showed significantly higher values for EIAab kit; in extreme cases several scores higher than those obtained with R&D kit. These findings highlight the role of a kind of assay used for the TSLP measurement for the results obtained. The greatest differences between the results of the two kits used in this study were found in patients with high serum and sputum TSLP level. Although the B&A plots suggest a somehow better agreement between the results of the two kits in EBC, it ought to be underscored that TSLP levels were below the detection limit in many EBC samples and when detectable these levels were relatively low. In all B&A plots the distribution of points representing individual samples shows a proportional constant error; the higher the TSLP concentration, the greater difference between the EIAab and R&D results might be expected. The B&A plot, however, only defines the intervals of agreement; it does not stipulate what limit is acceptable or not. The acceptable limit should be defined *a priori*, based on clinical judgment,

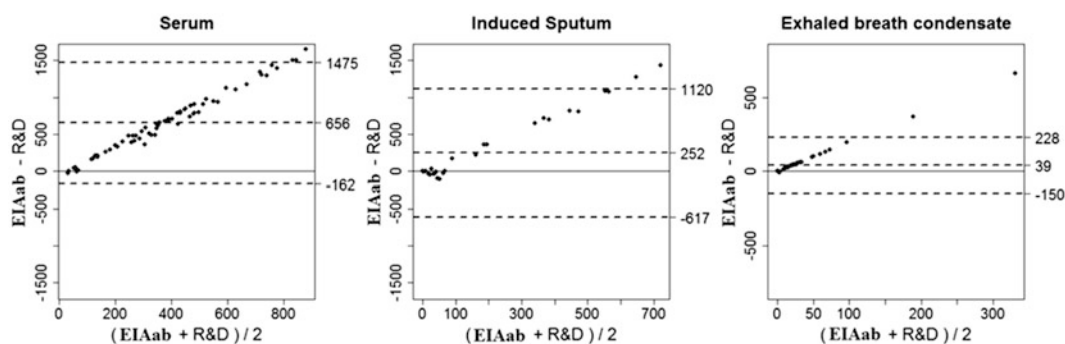


Fig. 1 The Bland–Altman plots showing the agreement between TSLP concentration in serum, induced sputum and exhaled breath condensate measured by R&D and EIAab kits. The Y axis: difference between EIAab and R&D measurements (EIAab–R&D; pg/mL),

the X axis: mean of EIAab and R&D measurements ((EIAab + R&D)/2; pg/mL). The middle dashed line in each plot represents the mean value, the border dashed lines represent 95 % confidence interval

biological considerations, or other goals (Francq and Govaerts 2016; Giavarina 2015).

In the present study, TSLP in EBC could be detected only with EIAab kit. All measurements performed with R&D kit were below the detection limit. These results are different than those recently reported by Glück et al. (2016) who have found similar TSLP levels in both EBC and serum. The different results may have to do with the patients' characteristics and the methods of TSLP measurement. Only were patients with mild-to-moderate asthma, untreated with anti-inflammatory agents, enrolled into the present study, while the other study has evaluated asthma patients treated with steroids, antileukotrienes, and theophylline. In both studies, serum and EBC TSLP levels were measured by R&D ELISA kit. However, Glück et al. (2016) have included in the analysis the TSLP measurements reaching the half-value of the detection limit, quantified by extrapolation of standard assay curves, while we analyzed only the TSLP values that exceeded the detection limit. A low level of TSLP in EBC demonstrated in the present study can be related either to low activity of cellular TSLP sources releasing this protein to EBC or to high activity of its local degradation. Low TSLP may also indicate that its local epithelial production and systemic release are independent from each other. However, scarcity of data does not allow drawing hard conclusions on the origin and level of TSLP in EBC.

We also demonstrate that, regardless of the ELISA kit used, TSLP level in IS was significantly higher in asthmatics than in COPD patients. Since sputum TSLP in asthma and COPD patients has not yet been studied, we could not confront our results with other studies. As TSLP is linked to Th-2 immune response, a higher level of sputum TSLP in asthmatics might be explained by the nature of allergic airway inflammation. In this context, our results are consistent with those reported by Ying et al. (2005), who have found a higher level of TSLP in bronchial biopsies from asthmatics compared with control subjects. In earlier studies, elevated TSLP concentration in asthma patients has also been found in the serum (Glück et al. 2016). In

contrast, the present study demonstrates similar serum TSLP levels in patients with asthma, COPD, and in control subjects. Only was the serum TSLP measured by R&D kit comparable with that published by other authors, while TSLP measured by EIAab kit was app. 4–20-fold higher than that reported earlier (Glück et al. 2016). It might be posited that the lack of differences between serum TSLP in asthma and COPD was related to cigarette smoking by COPD patients. Smoking is associated with oxidative stress, which enhances TSLP expression (Ying et al. 2008). Thus, despite different underlying pathomechanisms, Th-2 allergic response in asthmatics and cigarette smoking in COPD patients, TSLP would be similar in both groups. On the other hand, if oxidative stress led to increased TSLP expression, higher levels should be expected not only in the serum of COPD patients but also in IS. That was not the case in this study. Similar serum TSLP levels in patients with obstructive airway diseases (asthma and COPD) and in control subjects might be even more difficult to explain. It should be noted that there were two smokers in the control group, but it seems highly unlikely that their results could significantly affect the serum TSLP level. A comparative analysis of TSLP level in IS and serum measured by R&D kit in the three investigated groups leads to the following hypothesis: if allergic airway inflammation is associated with increased TSLP expression, its level could be more reliably reflected in IS than in the serum.

The difference between the results of TSLP obtained with R&D and EIAab kits might be due to different antibodies present in both kits. The antibody is a major factor determining sensitivity and specificity of an assay. The three-dimensional configuration of the antigen-binding site of an antibody controls the strength of an interaction with the antigen. Additionally, a competing factor is cross-reactivity of the antibody with proteins other than the target antigen, which has to do with the antibodies being polyclonal or monoclonal. In either case, driving the assay to the limit of sensitivity may result in cross-reactivity, and the conflicting needs of sensitivity

and specificity. Unfortunately, we could not find specific data on antibodies used in the two ELISA kits used in this study. One of the limitations of ELISA technique is that it provides data on the presence of an analyte but no information on its biochemical properties. It is known that alternative splicing of the *TSLP* gene results in two transcript variants; hence either TSLP isoform should be analyzed separately (Fornasa et al. 2015).

This study has some limitations. The number of patients was relatively low, and this refers particularly to control subjects. Although the serum and EBC samples were taken from almost all patients, IS was obtained from only 30 out of the 72 subjects, since some patients were unable to expectorate sputum or its quality was inadequate (e.g., it contained too many epithelial cells). The enrolment of patients with only mild-to-moderate disease and the lack of analysis of the relationship between specific asthma and COPD features (e.g., atopic vs. non-atopic or controlled vs. uncontrolled) might also be considered a limitation of the study.

5 Conclusions

The results of TSLP measurement in various biological samples are highly dependent on the ELISA assay used. Thus, comparison of results obtained with different assays may be confusing and may lead to wrong conclusions. The sputum TSLP seems a more reliable estimator in asthma than serum TSLP. Since we found an appreciably higher sputum TSLP level in asthma than in COPD or control subjects, we submit that the sputum TSLP might be potentially used as asthma biomarker.

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

References

Cook EB, Stahl JL, Schwantes EA, Fox KE, Mathur SK (2012) IL-3 and TNF α increase Thymic Stromal

- Lymphopoietin Receptor (TSLPR) expression on eosinophils and enhance TSLP-stimulated degranulation. *Clin Mol Allergy* 10:8
- Djukanović R, Sterk PJ, Fahy JV, Hargreave FE (2002) Standardised methodology of sputum induction and processing. *Eur Respir J Suppl* 37:1s–2s
- Ferreira MAR, Matheson MC, Tang CS et al (2014) Genome-wide association analysis identifies 11 risk variants associated with the asthma with hay fever phenotype. *J Allergy Clin Immunol* 133:1564–1571
- Fornasa G, Tsilingiri K, Caprioli F et al (2015) Dichotomy of short and long thymic stromal lymphopoietin isoforms in inflammatory disorders of the bowel and skin. *J Allergy Clin Immunol* 136:413–422
- Franco BG, Govaerts B (2016) How to regress and predict in a Bland-Altman plot? Review and contribution based on tolerance intervals and correlated-errors-in-variables models. *Stat Med* 35:2328–2358
- Gauvreau GM, O'Byrne PM, Boulet LP et al (2014) Effects of an anti-TSLP antibody on allergen-induced asthmatic responses. *N Engl J Med* 370:2102–2110
- Giavarina D (2015) Understanding Bland-Altman analysis. *Biochem Medica* 25:141–151
- GINA (2015). Global strategy for asthma management and prevention. <http://www.ginasthma.org>. Accessed on 9 May 2016
- Glück J, Rymarczyk B, Kasprzak M, Rogala B (2016) Increased levels of interleukin-33 and thymic stromal lymphopoietin in exhaled breath condensate in chronic bronchial asthma. *Int Arch Allergy Immunol* 169:51–56
- GOLD (2015). Global initiative for chronic obstructive lung disease. http://www.goldcopd.it/materiale/2015/GOLD_Report_2015.pdf. Accessed 9 May 2016
- Górka K, Maskey-Warzechowska M, Nejman-Gryz P, Korczyński P, Prochorec-Sobieszek M, Krenke R (2016) Comparative study of periostin expression in different respiratory samples in patients with asthma and chronic obstructive pulmonary disease. *Pol Arch Med Wewn* 126:124–137
- Gudbjartsson DF, Bjornsdottir US, Halapi E et al (2009) Sequence variants affecting eosinophil numbers associate with asthma and myocardial infarction. *Nat Genet* 41:342–347
- Harada M, Hirota T, Jodo AI et al (2011) Thymic stromal lymphopoietin gene promoter polymorphisms are associated with susceptibility to bronchial asthma. *Am J Respir Cell Mol Biol* 44:787–793
- Horváth I, Hunt J, Barnes PJ et al (2005) Exhaled breath condensate: methodological recommendations and unresolved questions. *Eur Respir J* 26:523–548
- Hunninghake GM, Lasky-Su J, Soto-Quirós ME et al (2008) Sex-stratified linkage analysis identifies a female-specific locus for IgE to cockroach in Costa Ricans. *Am J Respir Crit Care Med* 177:830–836
- Ito T, Liu Y-J, Arima K (2012) Cellular and molecular mechanisms of TSLP function in human allergic disorders – TSLP programs the ‘Th2 code’ in dendritic cells. *Allergol Int* 61:35–43

- Kato A, Favoreto S, Avila PC, Schleimer RP (2007) TLR3- and Th2 cytokine-dependent production of thymic stromal lymphopoietin in human airway epithelial cells. *J Immunol* 179:1080–1087
- Lee HC, Ziegler SF (2007) Inducible expression of the proallergic cytokine thymic stromal lymphopoietin in airway epithelial cells is controlled by NF-kappaB. *Proc Natl Acad Sci U S A* 104:914–919
- Lee EB, Kim KW, Hong JY, Jee HM, Sohn MH, Kim KE (2010) Increased serum thymic stromal lymphopoietin in children with atopic dermatitis. *Pediatr Allergy Immunol* 21:e457–e460
- Leonard WJ (2002) TSLP: finally in the limelight. *Nat Immunol* 3:605–607
- Levin SD, Koelling RM, Friend SL, Isaksen DE, Ziegler SF, Perlmutter RM, Farr AG (1999) Thymic stromal lymphopoietin: a cytokine that promotes the development of IgM+ B cells in vitro and signals via a novel mechanism. *J Immunol* 162:677–683
- Liu YJ (2006) Thymic stromal lymphopoietin: master switch for allergic inflammation. *J Exp Med* 203:269–273
- Liu YJ, Soumelis V, Watanabe N, Ito T, Wang YH, Malefyt Rde W, Omori M, Zhou B, Ziegler SF (2007) TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. *Annu Rev Immunol* 25:193–219
- Noti M, Wojno EDT, Kim BS et al (2013) Thymic stromal lymphopoietin-elicited basophil responses promote eosinophilic esophagitis. *Nat Med* 19:1005–1013
- Pandey A, Ozaki K, Baumann H, Levin SD, Puel A, Farr AG, Ziegler SF, Leonard WJ, Lodish HF (2000) Cloning of a receptor subunit required for signaling by thymic stromal lymphopoietin. *Nat Immunol* 1:59–64
- Park LS, Martin U, Garka K et al (2000) Cloning of the murine thymic stromal lymphopoietin (TSLP) receptor: formation of a functional heteromeric complex requires interleukin 7 receptor. *J Exp Med* 192:659–670
- Pellegrino R, Viegi G, Brusasco V et al (2005) Interpretative strategies for lung function tests. *Eur Respir J* 26:948–968
- Quentmeier H, Drexler HG, Fleckenstein D, Zaborski M, Armstrong A, Sims JE, Lyman SD (2001) Cloning of human thymic stromal lymphopoietin (TSLP) and signaling mechanisms leading to proliferation. *Leukemia* 15:1286–1292
- Reche PA, Soumelis V, Gorman DM et al (2001) Human thymic stromal lymphopoietin preferentially stimulates myeloid cells. *J Immunol* 167:336–343
- Sokol CL, Barton GM, Farr AG, Medzhitov R (2008) A mechanism for the initiation of the Th2 response by an allergen. *Nat Immunol* 9:310–318
- Vignola AM, Rennar SI, Hargreave FE, Fah JV, Bonsignore MR, Djukanović R, Sterk PJ (2002) Standardised methodology of sputum induction and processing. Future directions. *Eur Respir J Suppl* 37:51s–55s
- Watanabe J, Saito H, Miyatani K, Ikeguchi M, Umekita Y (2015) TSLP expression and high serum TSLP level indicate a poor prognosis in gastric cancer patients. *Yonago Acta Med* 58:137–143
- Watson B, Gauvreau GM (2014) Thymic stromal lymphopoietin: a central regulator of allergic asthma. *Expert Opin Ther Targets* 18:771–785
- Ying S, O'Connor B, Ratoff J, Chen S (2005) Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity. *J Immunol* 174:8183–8190
- Ying S, O'Connor B, Ratoff J et al (2008) Expression and cellular provenance of thymic stromal lymphopoietin and chemokines in patients with severe asthma and chronic obstructive pulmonary disease. *J Immunol* 181:2790–2798
- Ying G, Zhang Y, Tang G, Chen S (2015) Functions of thymic stromal lymphopoietin in non-allergic diseases. *Cell Immunol* 295:144–149
- Zhang K, Shan L, Rahman MS, Unruh H, Halayko AJ, Gounni AS (2007) Constitutive and inducible thymic stromal lymphopoietin expression in human airway smooth muscle cells: role in chronic obstructive pulmonary disease. *Am J Physiol Lung Cell Mol Physiol* 293:L375–L382
- Zhou B, Comeau MR, De Smedt T, Liggitt HD, Dahi ME, Lewis DB, Gyarmati D, Aye T, Campbell DJ, Ziegler SF (2005) Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. *Nat Immunol* 6:1047–1053

Prevalence of *Neisseria meningitidis* Carriage with Identification of Serogroups and Genogroups in Professional Soldiers

K. Korzeniewski, M. Konior, M. Kiedrowska, E. Wódka,
E. Zwolińska, and A. Skoczyńska

Abstract

The article presents the prevalence of *Neisseria meningitidis* carriage with the identification of sero- and genogroups in professional soldiers serving in the Polish Armed Forces. A total of 1246 soldiers from the 10th Armored Cavalry Brigade in Świętoszów, Poland were examined in the period January–February 2016. Microbiological tests were performed using standard methods (culture, incubation, microscopy, biochemical, and automated identification with VITEK cards). *Neisseria meningitidis* isolates from carriers were subjected to a slide agglutination test for the identification of serogroups, next bacterial DNA was isolated and genogroups were identified based on the results of PCR. Of the 1246 soldiers tested, 65 were found to be carriers of *N. meningitidis*. Serogroups of 36 isolates and genogroups of 56 meningococcal isolates were determined. The genogrouping identified the isolates as belonging to group B (n = 34; 52.3 %), E29 (n = 8; 12.3 %), C (n = 6; 9.2 %), Y (n = 6; 9.2 %), and W (n = 2; 3.1 %). The primers which were used did not make it possible to determine the genogroup of nine isolates. In conclusion, the overall carrier rate of *N. meningitidis* amounted to 5.2 %, with the serogroup B being predominant, which is similar to that reported in the general population in Poland and Central Europe.

Keywords

Neisseria meningitidis • Serogrouping • Genogrouping • Carriage • Prevalence • Soldiers

K. Korzeniewski (✉) and M. Konior
Department of Epidemiology and Tropical Medicine,
Military Institute of Medicine, 128 Szaserów Street, 04-
141 Warszawa, Poland
e-mail: kktropmed@wp.pl

M. Kiedrowska and A. Skoczyńska
National Reference Center for Bacterial Meningitis,
National Medicines Institute, Warsaw, Poland

E. Wódka
Department of Medical Diagnostics, Military Institute of
Medicine, Warsaw, Poland

E. Zwolińska
Department of Gynecology, Holy Family Maternity
Hospital, Warsaw, Poland

1 Introduction

Neisseria meningitidis (*N. meningitidis*) is a major etiological factor associated with infections of the central nervous system (CNS). CNS infections commonly take the form of meningitis or sepsis and are referred to as invasive meningococcal disease (Rosenstein et al. 2001). The bacteria colonize the nasopharyngeal mucosa asymptomatically and *N. meningitidis* carriers are the major source of infection (Soriano-Gabarro et al. 2011). Estimated prevalence of *N. meningitidis* in the general population ranges from 5 to 10 % (Skoczyńska and Hryniewicz 2012), whereas in closed environments such as prisons, boarding schools, or military camps, meningococcal carriage rate may reach 40–80 % (Tyski et al. 2000). There are 12 serogroups of meningococcal strains described as A, B, C, E29, H, I, K, L, W135, X, Y, and Z; the serogroups are determined by the biochemical composition of a polysaccharide capsule of *N. meningitidis*. Serogroups A, B, C, Y, and W135 are most commonly isolated from carriers or in invasive infections. In 30–45 % of cases, it is impossible to identify the bacterial serogroup (Caugant et al. 2007; Bennett and Cafferkey 2006). Serogroups B and C are a major causes of meningococcal infections in North and South America as well as in Europe, while serogroups A and C account for a majority of infections in Asia and Africa. In recent years, especially in the United States, the United Kingdom, Sweden and Finland, the incidence of meningococcal infections caused by serogroup Y has increased (Skoczyńska and Hryniewicz 2012; Rosenstein et al. 2001). In general, serogroup prevalence varies between countries and changes over time.

Invasive meningococcal disease (IMD) remains one of the most serious infectious illnesses in the world, despite the use of antibiotics at an early stage and the development of intensive care facilities. IMD is an acute illness which may lead to death in less than 24 h. Mortality from IMD reaches 10–13 %, and in case of a septic shock – as much as 70 %

(Skoczyńska and Hryniewicz 2012; Caugant et al. 1994). Two hundred and eighteen cases of the diseases have been reported in Poland in 2015 (National Institute of Public Health 2015), and there were 103 deaths from IMD between 2010 and 2014 (KOROUN 2015). The IMD outbreaks are rare in the Polish Armed Forces, yet they may pose a significant health hazard. In 2006, four microbiologically confirmed IMD cases were reported in a military unit in Skwierzyna; two of the infected soldiers died (Grecki and Bienias 2006). In 2007, another 15 cases of IMD were reported from a military airbase in Warsaw; two of the patients died (Kadłubowski et al. 2007). Between 2006 and 2008, there were reports of microbiologically confirmed IMD cases from military units in the cities of Wrocław, Gliwice, Gołdap, Warszawa-Wesoła, Toruń, Koszalin, and Przemyśl. In December 2011, a Polish soldier died from IMD while serving in Afghanistan. Although there have been a number of confirmed cases of IMD among soldiers, some of which were fatal, there have been few studies into the prevalence of *N. meningitidis* carriage in the military environment. The studies by Tyski et al. (2001) conducted in 1998 and 1999 have reported that out of the 151 and 168 soldiers tested, 36 % and 61 %, respectively, were carriers of *N. meningitidis*. These studies, however, involved conscripts and Poland suspended conscription in 2009. The only study on *N. meningitidis* carriage in professional soldiers serving in the Polish Armed Forces was conducted in 2013 by research staff from the Department of Epidemiology and Tropical Medicine of the Military Institute of Medicine as part of the department's statutory activity. The study, involving 559 soldiers from the 25th Brigade stationed in the city of Tomaszów Mazowiecki, the unit in which the soldier who died from IMD in Afghanistan in 2011 had served, has found that 5.7 % of the soldiers tested were carriers of *N. meningitidis*. In non-vaccinated soldiers ($n = 302$), the carriage rate was 9.6 %, whereas among the vaccinated individuals ($n = 257$) it was only 1.2 %. The carriage rate was thus eight-fold lower in the vaccinated soldiers

than that in the non-vaccinated ones, which suggests that vaccination is an effective method of inducing herd immunity (Korzeniewski et al. 2015). To investigate whether it is necessary to vaccinate all the Polish military personnel with a quadrivalent conjugate vaccine against *N. meningitidis* serogroups A, C, Y, and W135 and with a newly introduced vaccine against serogroup B, we were obliged to conduct a large-scale population study to investigate the prevalence of *N. meningitidis* carriage in the military environment. The obligation was in compliance with a regulation of the Minister of Defense of February 2014 on a vaccination schedule for professional soldiers. Therefore, the aim of the present study was to assess the prevalence of *N. meningitidis* carriage and to identify the sero- and genogroups in professional soldiers serving in the Polish Armed Forces.

2 Methods

The research task was approved by the Ethics Committee of the Military Institute of Medicine in Warsaw, Poland (permit 24/WIM/2014 of 18 Aug 2014).

2.1 Study Population

We tested 1246 professional soldiers from the 10th Armored Cavalry Brigade stationed in the city of Świątoszów after they had provided informed consent and completed a questionnaire concerning personal information such as military rank, age, gender, place of residence, cigarette smoking, symptoms of a respiratory tract infection, medications used on a regular basis, and vaccinations against meningococcal infections. The inclusion criteria were as follows: age of 20–55 years and a good general health, with a possible respiratory tract infection but no pathological lesions in the nasopharynx that would make it impossible to take a swab sample. The biological material (nasopharyngeal swabs) was taken during the winter season (January–

February) of 2016 on the premises of the military unit.

2.2 Laboratory Workup

Identification of Isolates The cultures were inoculated, using a streaking procedure, onto the Columbia Agar medium with 5 % sheep blood and PoliVitex VCA3 and incubated under elevated CO₂ concentration at 37 °C for 48 h. They were then transported to a microbiological laboratory of the Military Institute of Medicine in Warsaw where the colonies grown were macroscopically evaluated. The colonies morphologically similar to *N. meningitidis* strains were isolated onto Columbia Agar with 5 % sheep blood plates, and were then incubated under elevated CO₂ concentration at 37 °C for another 24–48 h. Catalase and cytochrome oxidase tests were performed. Gram-stained preparations were examined with light microscopy. All catalase and cytochrome oxidase-positive strains, as well as strains morphologically similar to Gram-negative cocci (microscopic evaluation) were then identified with biochemical tests. The identification was carried out by means of API NH biochemical sets and an automated system for identification of microorganisms using Vitek 2 NH cards (bioMérieux; Marcy l'Etoile, France). The strains identified as *N. meningitidis* were stored at –20 °C and further transported to the National Reference Center for the Diagnostics of Bacterial Infections of Central Nervous System (KOROUN) in Warsaw, Poland for re-identification, serogrouping, DNA isolation, and genogrouping.

Serogrouping The strains delivered to KOROUN were revived by placing them onto the Columbia Agar medium; they were incubated in elevated CO₂ atmosphere at 37 °C for 24 h. Serogroups of the isolates were identified with a slide agglutination test using a set of primers according to the manufacturer's recommendations. The serogroup-specific reagents included the serogroups: A, B, C, Y, and W (Thermo

Fisher Scientific, Remel Products; Lenexa, KS), E29 (Bio-Rad Laboratories LTD; Hemel Hempstead, UK), and X and Z (Becton Dickinson; Franklin Lakes, NJ).

DNA Isolation Chromosomal DNA was isolated from meningococcal isolates with Genomic DNA Prep Plus (A&A Biotechnology; Gdynia, Poland), following the manufacturer's recommendations.

Genogrouping Genogroups were identified with PCR assays using the genogroup-specific oligonucleotide primers *orf-2*(A), *siaD*(C), *siaD*(W135), and *siaD*(Y) according to the description of Taha (2000) and *siaD*(B) according to Guiver et al. (2000).

2.3 Statistical Elaboration

Quantitative variables were characterized by the arithmetic mean \pm SD or median with max/min range and 95 % confidence interval. Qualitative variables were presented as count and percentage. To check if a quantitative variable derives from a population of normal distribution the Shapiro-Wilk test was used. The Leven test was used to demonstrate the homogeneity of variances. Statistical significance of differences between two groups was processed with a *t*-test or Mann-Whitney *U* test. A logistic model was used to examine dependency of socio-demographic variables and the non-carrier/carrier groups. A *p*-value under 0.05 was considered statistically significant. The

analyses were performed using a commercial STATISTICA package of StatSoft Inc. ver. 12.0.

3 Results

Out of the 1246 professional soldiers tested, 65 (5.2 %) were found to be carriers of *N. meningitidis*. The serogroups were determined for 36 *N. meningitidis* isolates. Five serogroups were identified: B (*n* = 25, 38.5 %), Y (*n* = 4, 6.15 %), E29 (*n* = 2, 3.1 %), C (*n* = 2, 3.1 %), W (*n* = 2, 3.1 %), and A (*n* = 1, 1.5 %). A large number of isolates polyagglutinated (*n* = 23, 35.4 %), other isolates autoagglutinated (*n* = 5, 7.7 %), and one isolate (1.5 %) did not agglutinate. The genogroups were determined for 56 isolates: B (*n* = 34, 52.3 %), E29 (*n* = 8, 12.3 %), C (*n* = 6, 9.2 %), Y (*n* = 6, 9.2 %), and W (*n* = 2, 3.1 %). It was impossible to determine the genogroup of nine isolates (NG, 13.8 %) with the primers used (Fig. 1). The results of serogrouping and genogrouping are presented in Table 1.

The mean age of *N. meningitidis* carriers was 30.7 ± 5.0 years (range 21–45 years) and that of non-carriers was 31.7 ± 5.4 years (range 20–59 years); the difference between the two groups was insignificant. There was one woman in the carrier group and 116 women (9.8 %) in the non-carrier group; the proportion of women among the non-carriers was significantly higher (*p* = 0.044). As for the carrier group, the proportion of subjects living in rural areas was 38.5 %, which was grossly akin to that in the non-carrier group of 40.9 %. There were no significant

Fig. 1 Percentage distribution of carriers of the *N. meningitidis* genogroups B, E29, C, Y, and W in the soldiers examined (*n* = 65) (NG isolates that could not be allocated to any specific genogroup with the primers used)

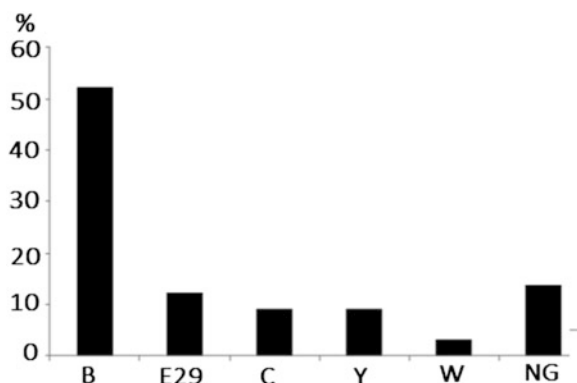


Table 1 Serogrouping (monoclonal serum), presented in rows, and genogrouping (PCR), presented in columns, of *N. meningitidis* isolates (n = 65)

Sero/Genogrouping	A	B	C	E29	W	Y	NG	Total
A	–	–	–	–	–	–	1	1
Autoagglutination	–	2	–	1	–	–	2	5
B	–	25	–	–	–	–	–	25
C	–	–	2	–	–	–	–	2
E29	–	–	–	2	–	–	–	2
Polyagglutination	–	7	4	5	–	2	5	23
NG	–	–	–	–	–	–	1	1
W	–	–	–	–	2	–	–	2
Y	–	–	–	–	–	4	–	4
Total	0	34	6	8	2	6	9	65

NG isolates that could not be allocated to any specific genogroup with the primers used

Table 2 Socio-demographic variables of non-carriers and carriers of *N. meningitidis*

Socio-demographics	Non-carriers (n = 1181)	Carriers (n = 65)	p-value
Age (years)			
Mean (SD)	31.7 (5.4)	30.7 (5.0)	0.188
Range	20.0–59.0	21.0–45.0	
Median	31.0	31.0	
95%CI	31.4–32.0	29.5–32.0	
Gender			
Women	116 (9.8 %)	1 (1.5 %)	0.044
Men	1065 (90.2 %)	64 (98.5 %)	
Place of residence			
Rural area	483 (40.9 %)	25 (38.5 %)	0.693
Urban area	697 (59.1 %)	40 (61.5 %)	
Cigarette smoking			
Yes	396 (33.5 %)	30 (46.2 %)	0.037
No	785 (66.5 %)	35 (53.8 %)	
Respiratory tract infection			
Yes	86 (7.3 %)	2 (3.1 %)	0.198
No	1095 (92.7 %)	63 (96.9 %)	
Military rank			
Private	742 (62.8 %)	47 (72.3 %)	0.087
Non-commissioned officer	371 (31.4 %)	18 (27.7 %)	
Commissioned officer	68 (5.8 %)	0 (0 %)	
Vaccinated			
Yes	178 (15.1 %)	6 (9.2 %)	0.196
No	1003 (84.9 %)	59 (90.8 %)	

correlations between the place of residence and the study group ($p = 0.693$). The proportion of smokers in the carrier group was 46.2 %, which was significantly greater than the 33.5 % in the non-carrier group ($p = 0.037$). The proportion of privates in the carrier group was 72.3 % compared with 62.8 % in the non-carrier group; the proportion of non-commissioned officers (NCO) was 27.7 % and 31.4 % and the proportion of

commissioned officers (CO) was 0.0 % and 5.8 % in the respective groups. There were no significant differences in the distribution of military ranks between the two study groups ($p = 0.087$). The proportion of vaccinated soldiers in the *N. meningitidis* carrier group was 9.2 % and in the non-carrier group it was 15.1 %, with no significant difference between the two groups ($p = 0.196$) (Table 2).

In the *N. meningitidis* non-carrier group, the mean age of the vaccinated soldiers was 33.5 ± 4.8 years (range 23–49 years) and of the non-vaccinated ones it was 31.4 ± 5.4 years (range 20–59 years); the latter were significantly younger ($p = 0.0001$). The proportion of women among the vaccinated soldiers was 2.7 % and among the non-vaccinated ones it was 10.5 %; the difference was significant, $p = 0.0008$. The proportion of privates among the vaccinated soldiers was 45.1 %, and among the non-vaccinated ones it was 66.5 % (the difference was significant, $p = 0.0001$) (Table 3).

In the *N. meningitidis* carrier group, the mean age of the vaccinated soldiers was 34.5 ± 2.6 years (range 32–38 years) and of the non-vaccinated ones it was 30.3 ± 5.0 years (range 21–45 years); the latter were significantly younger ($p = 0.025$). There were no women in the group of vaccinated soldiers; the proportion of women in the group of non-vaccinated soldiers was 1.7 %. No significant differences were found between gender and vaccination ($p = 0.156$). The proportion of privates among

the vaccinated soldiers was 0.0 %, and among the non-vaccinated soldiers it was 79.7 % (the difference was significant, $p = 0.0002$) (Table 4).

The logistic regression analysis (univariate and multivariate) identified two factors significantly associated with increased risk of *N. meningitidis* carriage, i.e., smoking cigarettes and low military rank (Table 5).

4 Discussion

The only natural habitat of *N. meningitidis* in humans is the nasopharyngeal mucosa. The bacteria colonize the nasopharynx and spread through inhalation of droplets of respiratory secretions or through a direct contact with a carrier (e.g., sharing a drink or a cigarette with an infected person). In most cases, meningococcal carriage does not lead to invasive disease. Yet it may cause an invasive meningococcal disease (IMD) under certain conditions such as concomitant respiratory tract infections, dental diseases,

Table 3 Socio-demographic variables among soldiers vaccinated with quadrivalent vaccine A, C, W135, and Y, and non-vaccinated soldiers in the group of *N. meningitidis* non-carriers

Socio-demographics	Vaccinated (n = 184)	Non-vaccinated (n = 1062)	p-value
Age (years)			
Mean (SD)	33.5 (4.7)	31.3 (5.4)	0.0001
Range	23.0–49.0	20.0–59.0	
Median	34.0	31.0	
95%CI	32.8–34.2	31.0–31.6	
Gender			
Women	5 (2.7 %)	112 (10.5 %)	0.001
Men	179 (97.3 %)	950 (89.5 %)	
Place of residence			
Rural area	68 (37.0 %)	440 (41.5 %)	0.250
Urban area	116 (63.0 %)	621 (58.5 %)	
Cigarette smoking			
Yes	79 (42.9 %)	347 (32.7 %)	0.007
No	105 (57.1 %)	715 (67.3 %)	
Respiratory tract infection			
Yes	16 (8.7 %)	72 (6.8 %)	0.349
No	168 (91.3 %)	990 (93.2 %)	
Military rank			
Private	83 (45.1 %)	706 (66.5 %)	0.0001
Non-commissioned officer	89 (48.4 %)	300 (28.2 %)	
Commissioned Officer	12 (6.5 %)	56 (5.3 %)	

Table 4 Socio-demographic variables among soldiers vaccinated with quadrivalent vaccine A, C, W135, and Y, and non-vaccinated soldiers in the group of *N. meningitidis* carriers

Socio-demographics	Vaccinated (n = 6)	Non-vaccinated (n = 59)	p-value
Age (years)			
Mean (SD)	34.5 (2.6)	30.3 (5.0)	0.025
Range	32.0–38.0	21.0–45.0	
Median	34.0	30.0	
95%CI	31.8–37.2	29.0–31.6	
Gender			
Women	0 (0.0 %)	1 (1.7 %)	0.156
Men	6 (100.0 %)	58 (98.3 %)	
Place of residence			
Rural area	1 (16.7 %)	24 (40.7 %)	0.477
Urban area	5 (83.3 %)	35 (59.3 %)	
Cigarette smoking			
Yes	2 (33.3 %)	28 (47.5 %)	0.817
No	4 (66.7 %)	31 (52.5 %)	
Respiratory tract infection			
Yes	0 (0 %)	2 (3.4 %)	0.434
No	6 (100 %)	57 (96.6 %)	
Military rank			
Private	0 (0 %)	47 (79.7 %)	0.0002
Non-commissioned officer	6 (100 %)	12 (20.3 %)	
Commissioned officer	0 (0 %)	0 (0 %)	

Table 5 Factors associated with increased risk for *N. meningitidis* carriage according to the logistic regression analysis (univariate and multivariate)

Socio-demographics	Logistic regression – univariate			Logistic regression – multivariate		
	Assessment	Odds ratio	p-value	Assessment	Odds ratio	p-value
Age	–0.1	1.0	0.163	–0.1	1.0	0.341
Gender						
Men	1.0	2.6	0.055	1.0	2.7	0.050
Women	–1.0	0.4	0.055	–1.0	0.3	0.050
Place of residence						
Rural area	–0.1	1.0	0.693	–0.1	0.9	0.578
Urban area	0.1	1.1	0.693	0.1	0.1	0.578
Cigarette smoking						
Yes	0.3	1.3	0.039	0.3	1.3	0.042
No	–0.3	0.8	0.039	–0.3	0.8	0.042
Respiratory tract infection						
Yes	–0.5	0.6	0.213	–0.5	0.6	0.255
No	0.5	1.6	0.213	0.5	1.6	0.207
Military rank						
Private	5.8	340.6	0.0010	5.7	290.4	0.0001
Non-commissioned officer	4.9	133.9	0.0001	4.9	137.5	0.0001
Commissioned officer	–10.1	0.01	-	–9.9	0.1	-
Vaccinated						
Yes	–0.3	0.8	0.202	–0.3	0.7	0.050
No	0.3	1.3	0.202	0.3	1.3	0.187

and the like. Studies concerning the prevalence of carriage conducted among military recruits have shown that despite a high asymptomatic infection rate, hypervirulent *N. meningitidis* strains rarely colonize the nasopharyngeal mucosa (Tzanakaki et al. 1993; Caugant et al. 1988). In fact, epidemic strains are found only in 1.4–1.6 % of healthy individuals (Cartwright et al. 1987). In an outbreak of IMD, however, the prevalence of hypervirulent strains in carriers can be significantly higher (Edwards et al. 1977). *N. meningitidis* carrier rates among European recruits are high, regardless of the size of a given study group or country of origin. Andersen et al. (1998) have found that the carriage rate among 1069 Danish recruits was 39–47 %. The surveillance study conducted among 1179 German recruits has found that 32.6 % of the soldiers are carriers of *N. meningitidis* (Claus et al. 2005). In Norway, a meningococcal carriage study in 126 military recruits has demonstrated the prevalence of *N. meningitidis* carriage at 61.9 % (Caugant et al. 2007). By contrast, our previous investigation on meningococcal carriage, conducted in 559 professional Polish soldiers, has found that only 5.7 % of them were *N. meningitidis* carriers, which is similar to the overall meningococcal carriage rates in the general population. That study has also revealed that serogroup B was the most prevalent one among the carriers (28 %) (Korzeniewski et al. 2015). In fact, serogroup B is still the most common of all *N. meningitidis* strains isolated from soldiers in Europe. This group is the most frequently identified serogroup among recruits studied in France (46 %) (Chapalain et al. 1992), Poland (32 %) (Tyski et al. 2001), and Germany (42 %) (Claus et al. 2005). It is important that meningococcal carriage studies focus not only on the identification of serogroups but also on the risk factors shaping the *N. meningitidis* carriage rate. Smoking, both active and passive, is a major contributing factor for the nasopharyngeal carriage of *N. meningitidis* (Stuart et al. 1989). This was confirmed in the present study as the logistic regression analysis found that smoking was

associated with increased risk for meningococcal carriage.

Studies into the prevalence of meningococcal carriage in the military environment are widely available. However, the majority are limited to one type of community only, i.e., young, newly drafted recruits. Some European countries, including Poland, have suspended compulsory conscription and transformed their national armies into fully professional organizations. As a result of those changes, the mean age of soldiers has increased; 19–20 years old recruits are replaced by 25–30 years old professional privates. The past young recruits used to serve on a 24/7 basis, were accommodated in barracks, and had meals in military dining facilities; the conditions that are conducive to *N. meningitidis* carriage. The present time older professional soldiers, on the other hand, typically work eight hours a day and are not accommodated on the premises of a military unit, with the exception of rather infrequent 24-h duties, military exercises, or military operations. In fact, professional military service has changed from 24/7 service to a regular job, with risk factors similar to those found in the civilian environment. That is reflected by a relatively higher age of thirty odd years of soldiers in the present study; the age at which the prevalence of meningococcal carriage seems reduced.

5 Conclusions

The overall carrier rate of *N. meningitidis* in the study group was 5.2 %, with serogroup B being predominant (52.3 %), which is similar to the rate reported in the general population in Poland and Central Europe. Considering the fact that meningococcal carrier state may be chronic and last for several months, or it may be irregular and intermittent as shown by variability in colonization of the nasopharyngeal mucosa, it is necessary to conduct further research into the prevalence of *N. meningitidis* carriage among soldiers, especially in the context of the validity of introducing immunoprophylaxis against

meningococcal infections for the entire military personnel of the Polish Armed Forces.

Acknowledgments We are grateful to the Commander of the 10th Armored Cavalry Brigade in Świętoszów for permission to carry out this study and to the soldiers of this military unit for their participation in examination. The paper was supported by the Polish Ministry of Science and High Education, grant no. 326/2015.

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

References

- Andersen J, Barthelsen L, Bech Jensen B, Lind I (1998) Dynamics of the meningococcal carrier state and characteristics of the carrier strains: a longitudinal study within three cohorts of military recruits. *Epidemiol Infect* 121:85–94
- Bennett DE, Cafferkey MT (2006) Consecutive use of two multiplex PCR-based assays for simultaneous identification and determination of capsular status of nine common *Neisseria meningitidis* serogroups associated with invasive disease. *J Clin Microbiol* 44:1127–1131
- Cartwright KA, Stuart JM, Jones DM, Noah ND (1987) The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica*. *Epidemiol Infect* 99:591–601
- Caugant DA, Kristiansen BE, Frøholm LO, Bøvre K, Selander RK (1988) Clonal diversity of *Neisseria meningitidis* from a population of asymptomatic carriers. *Infect Immun* 56:2060–2068
- Caugant DA, Hoiby EA, Magnus P, Scheel O, Hoel T, Bjune G, Wedege E, Eng J, Frøholm LO (1994) Asymptomatic carriage of *Neisseria meningitidis* in a randomly sampled population. *J Clin Microbiol* 32(2):323–330
- Caugant DA, Tzanakaki G, Kriz P (2007) Lessons from meningococcal carriage studies. *FEMS Microbiol Rev* 31:52–63
- Chapalain JC, Guibourdenche M, Perrier-Gros-Claude JD, Bartoli M, Riou JY (1992) The chemoprophylaxis of cerebrospinal meningitis using rifampin in a military population. *Pathol Biol* 40:230–233
- Claus H, Maiden MC, Wilson DJ, McCarthy ND, Jolley KA, Urwin R, Hessler F, Frosch M, Vogel U (2005) Genetic analysis of meningococci carried by children and young adults. *J Infect Dis* 191:1263–1271
- Edwards EA, Devine LF, Sengbusch CH, Ward HW (1977) Immunological investigations of meningococcal disease. III. Brevity of group C acquisition prior to disease occurrence. *Scand J Infect Dis* 9:105–110
- Grecki M, Bienias M (2006) Outbreak of invasive meningococcal disease among soldiers in Skwierzyzna, Poland, March 2006. *Euro Surveill* 11(7):E060706.4
- Guiver M, Borrow R, Marsh J, Gray SJ, Kaczmarski EB, Howells D et al (2000) Evaluation of the Applied Biosystems automated Taqman polymerase chain reaction system for the detection of meningococcal DNA. *FEMS Immunol Med Microbiol* 28:173–179
- Kadłubowski M, Waśko I, Klarowicz A, Hryniewicz W (2007) Invasive meningococcal disease at a military base in Warsaw, January 2007. *Euro Surveill* 12(9): pii = 3147.
- KOROUN (2015) National reference center for the diagnostics of CNS infections. Warsaw, Accessed on 21 Mar 2016 (Article in Polish)
- Korzeniewski K, Skoczyńska A, Guzek A, Konior M, Chciałowski A, Waśko I, Markowska M, Zwolińska E (2015) Effectiveness of immunoprophylaxis in suppressing carriage of *Neisseria meningitidis* in the military environment. *Adv Exp Med Biol* 836:19–28
- National Institute of Public Health (2015) Infectious diseases and poisonings in Poland. National Institute of Hygiene, Department of Epidemiology. Department for Communicable Disease and Infection Prevention and Control, Chief of Sanitary Inspectorate, Warszawa, Accessed on 21 Mar 2016 (Article in Polish)
- Rosenstein NE, Perkins BA, Stephens DS, Popovic T, Hughes JM (2001) Meningococcal disease. *N Engl J Med* 344(18):1378–1388
- Skoczyńska A, Hryniewicz W (2012) Meningococcal infections. *Pol Merk Lek* 191:283–285 (Article in Polish)
- Soriano-Gabarro M, Wolter J, Hoge C, Vyse A (2011) Carriage of *Neisseria meningitidis* in Europe: a review of studies undertaken in the region. *Expert Rev Anti Infect Ther* 9(9):761–774
- Stuart JM, Cartwright KA, Robinson PM, Noah ND (1989) Effect of smoking on meningococcal carriage. *Lancet* 2(8665):723–725
- Taha MK (2000) Simultaneous approach for nonculture PCR-based identification and serogroup prediction of *Neisseria meningitidis*. *J Clin Microbiol* 38:855–857
- Tyski S, Grzybowska W, Dulny G (2000) Tests for *Neisseria meningitidis* in adolescents and adults (recruits). *Med Dosw Mikrobiol* 52:247–255 (Article in Polish)
- Tyski S, Grzybowska W, Dulny G, Berthelsen L, Lind I (2001) Phenotypical and genotypical characterization of *Neisseria meningitidis* carrier strains isolated from Polish recruits in 1998. *Eur J Clin Microbiol Infect Dis* 20:350–353
- Tzanakaki G, Blackwell CC, Kremastinou J (1993) Serogroups, serotypes and subtypes of *Neisseria meningitidis* isolated from patients and carriers in Greece. *J Med Microbiol* 38:19–22

Bacteriological Assessment of Pneumonia Caused by Gram-Negative Bacteria in Patients Hospitalized in Intensive Care Unit

A. Guzek, K. Korzeniewski, D. Tomaszewski, Z. Rybicki, and E. Zwolińska

Abstract

The article presents the results of 11-year study (2005–2015) of Gram-negative bacteria responsible for pneumonia in 2033 mechanically ventilated patients hospitalized in Intensive Care Unit. Of 8796 biological samples, consisting mainly of bronchial aspirate (97.9 %), 2056 bacterial strains were isolated and subjected to identification. VITEK 2 was used to determine drug susceptibility (classified according to the EUCAST criteria). ESBL, MBL and KPC-producing strains were identified by means of phenotypic methods using appropriate discs. The findings were that the predominant bacteria responsible for infections consisted of Enterobacteriaceae (42.0 %), *Acinetobacter baumannii* (37.2 %), *Pseudomonas aeruginosa* (16.1 %), and *Stenotrophomonas maltophilia* (4.7 %). We observed a rise in the number of bacteria causing pneumonia throughout the study period, especially in *S. maltophilia* and Enterobacteriaceae ESBL (+). Gram-negative bacilli were 100 % susceptible to colistin, apart from naturally resistant strains such as *Proteus mirabilis*, *Serratia marcescens*, whereas Enterobacteriaceae ESBL (+) were susceptible to imipenem and meropenem. *Acinetobacter baumannii* strains exhibited the lowest drug susceptibility. In conclusion, we report an increase in the prevalence of pneumonia associated with Gram-negative bacteria in mechanically ventilated intensive care patients. Colistin remains the most effective drug against the majority of Gram-negative bacteria. Therapeutic problems are common in the course of treatment of *Acinetobacter baumannii* infections.

A. Guzek
Department of Medical Diagnostics, Military Institute of
Medicine, Warsaw, Poland

K. Korzeniewski (✉)
Department of Epidemiology and Tropical Medicine,
Military Institute of Medicine, 128 Szaserów Street,
04-141 Warsaw, Poland
e-mail: kktropmed@wp.pl

D. Tomaszewski and Z. Rybicki
Clinic of Anesthesiology and Intensive Care, Military
Institute of Medicine, Warsaw, Poland

E. Zwolińska
Department of Gynecology, Holy Family Maternity
Hospital, Warsaw, Poland

Keywords

Bacteria identification • Bacterial strains • Pneumonia prevalence • Gram-negative bacteria • Antibiotics • Drug susceptibility • Intensive care

1 Introduction

Pneumonia accounts for the majority of infections in Intensive Care Units (ICU), especially in mechanically ventilated patients. Pneumonias may be classified in several ways. Infections acquired in a healthcare setting are referred to as nosocomial pneumonias (NP), these include hospital-acquired pneumonia (HAP). Infections that are not associated with healthcare are called community-acquired pneumonias (CAP), whereas those which result from mechanical ventilation are termed ventilator-associated pneumonias (VAP). VAP are present in 10–20 % of mechanically ventilated patients and are typically associated with an exceptionally high mortality rate reaching 70 %, depending on the type of causative bacteria (Kollef et al. 2005). Multicenter studies into the prevalence of infections in ICU, e.g., the European Prevalence of Infection in Intensive Care study (Vincent et al. 1995) or the International Study of the Prevalence and Outcomes of Infection in Intensive Care Units (Vincent et al. 2009), have shown that the respiratory failure associated with pneumonia amounts to 46.9–63.5 %. In recent years, there has been a large increase in the rate of nosocomial infections caused by multi-drug resistant Gram-negative bacteria, which is reflected in the growing prevalence of pneumonia caused by these microorganisms (Coque et al. 2008). The aim of the present study was to investigate the undergoing changes in prevalence of Gram-negative bacteria responsible for pneumonia in patients with an artificial airway ventilation hospitalized in ICU.

2 Methods

This research was approved by the Ethics Committee of the Military Institute of Medicine in Warsaw, Poland (permit no. 45/WIM/2014).

It is a retrospective study performed on 8796 biological samples obtained from 2033 patients

hospitalized in the Intensive Care Unit of the Military Institute of Medicine in Warsaw, Poland, between 2005 and 2015. A total of 2056 bacterial strains were isolated in the Microbiology Lab of the Institute. VITEK 2 automated system (bioMérieux; Marcy l'Etoile, France) was used to identify the species of isolates and to determine their antibiotic-susceptibility. The system was used in compliance with the manufacturer's instructions. Drug sensitivity of strains was interpreted according to the EUCAST (European Committee on Antimicrobial Susceptibility Testing) criteria. The reference strains *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 700603, and *Escherichia coli* ATCC 25922 were used as controls. Phenotypic tests were used to identify resistance of bacterial strains to antibiotics. Enterobacteriaceae, including extended spectrum beta-lactamase (ESBL)-producing strains, were identified using ceftazidime (30 µg)/cefotaxime (30 µg)/amoxicillin + clavulanic acid (20/10 µg) discs; the *Klebsiella pneumoniae* carbapenemase (KPC) enzyme was identified using meropenem (10 µg)/meropenem (10 µg) + boronic acid (300 µg) discs; and non-enterobacteriaceae metallo-beta-lactamase (MBL)-producing isolates were identified using ceftazidime (30 µg)/imipenem (10 µg)/ethylenediaminetetraacetic acid (EDTA) discs.

In statistical comparisons, an alpha level of 0.05 was used as a definer of significant differences. The analyses were performed using the commercial STATISTICA package of StatSoft Inc. ver. 10.0.

3 Results

A total of 2056 bacterial strains were isolated from the biological material (8796 samples) collected from 2330 patients hospitalized in the ICU between 2005 and 2015. The specimens included

Table 1 Material collected for microbiological examination in the period 2005–2015

Biological material	n (%)
Bronchial aspirate collected <i>via</i> a catheter through an artificial airway	8615 (97.9)
Bronchoalveolar lavage fluid	121 (1.4)
Pleural fluid	60 (0.7)
Total	8796 (100)

Table 2 The number and percentage of Gram-negative bacteria in the period 2005–2015

Gram-negative bacteria	No. of strains (%)
Enterobacteriaceae	864 (42.0)
<i>Acinetobacter baumannii</i>	764 (37.2)
<i>Pseudomonas aeruginosa</i>	332 (16.1)
<i>Stenotrophomonas maltophilia</i>	96 (4.7)
Total	2056 (100)

bronchial aspirate samples, bronchoalveolar lavage samples, and pleural fluid samples (Table 1).

97.9 % biological samples were collected *via* a catheter through an artificial airway (an endotracheal or tracheostomic tube). The samples were collected from each patient at multiple times, on average, 4.3 times. We did not assess repeating strains collected from the same patient, hence the difference between the number of samples collected ($n = 8796$) and the number of bacterial isolates identified ($n = 2056$). A great majority of our study subjects (87.4 %) showed clinical signs of respiratory failure and required mechanical ventilation. The remaining patients required an artificial airway ventilation due to a diminished cough reflex. All patients were mechanically ventilated for longer than 24 h. In line with the recommendations of the European Respiratory Society and the European Society of Clinical Microbiology and Infectious Diseases, pulmonary inflammation should be diagnosed on the basis of a chest radiograph or a physical examination and the presence of one of the following signs: purulent sputum, fever $>38^{\circ}\text{C}$, leukocytosis, or leukopenia (Torres et al. 2009). In the majority of the study patients pneumonia was caused by Enterobacteriaceae (42.0 %) and *Acinetobacter baumannii* (37.2 %) (Table 2).

Table 3 presents the exact number of bacterial isolates, and their numerical and percentage

growth in each study year, compared with 2005. Of all isolated Enterobacteriaceae as many 225 strains (10.9 %) were ESBL-producing Gram-negative bacilli. We observed a continued growth in the number of Enterobacteriaceae throughout the study period of 2005–2016, especially from 2012; for the ESBL-producing strains the growth was from 60 to 680 %. The number of KPC-producing strains was insignificant. An increase in the number of *Pseudomonas aeruginosa*-associated infections was found to be less pronounced smaller in comparison with the ESBL-producing Enterobacteriaceae (max. by 350 % in 2008, 270 % in 2011, and 150 % in 2015 as compared with 2005). The growth rate of *Acinetobacter baumannii* strains was similar. It is worth pointing out that there was a considerable increase in the number of *Stenotrophomonas maltophilia* strains, reaching 750 % in 2010 and 600 % in 2015.

The study demonstrate that all isolated Gram-negative bacilli were 100 % susceptible to colistin, except for naturally resistant strains such as *Proteus mirabilis* or *Serratia marcescens*. *Acinetobacter baumannii* strains exhibited the lowest therapeutic susceptibility rates. Table 4 shows the susceptibility and resistance of non-fermenting Gram-negative bacilli. The only effective therapeutic option for *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections was colistin. The susceptibility of *Pseudomonas aeruginosa* to ceftazidime and cefepime was more than 60 %, while *Acinetobacter baumannii* strains were found to be highly resistant to carbapenems; their therapeutic effectiveness was app. 35 %. Our study did not take into account the effect of sulbactam, as in Poland it is only available in combination with ampicillin and not in its pure form. Also, sulbactam's clinical efficacy has not been well documented, and the resistance to it develops rapidly when used.

The ESBL-producing Enterobacteriaceae exhibited a higher susceptibility to antibiotics. They were 100 % susceptible to imipenem and meropenem. On the other hand, KPC-producing *Klebsiella pneumoniae* strains were 100 % susceptible to colistin, tigecycline and gentamicin (Table 5).

Table 3 The number and percentage of Gram-negative bacteria between 2006 and 2015 as compared with 2005

Gram-negative bacteria	Total	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
<i>Enterobacteriaceae spp.</i>	864	38 (1.0)	60 (1.6)	73 (1.9)	69 (1.8)	69 (1.8)	69 (1.8)	74 (2.0)	98 (2.6)	91 (2.4)	101 (2.7)	122 (3.2)
ESBL (+)	225	5 (1.0)	20 (4.0)	20 (4.0)	5 (1.0)	9 (1.8)	16 (3.2)	8 (1.6)	35 (7.0)	36 (7.2)	39 (7.8)	32 (6.4)
ESBL (–)	637	33 (1.0)	40 (1.21)	53 (1.61)	64 (1.94)	60 (1.82)	53 (1.61)	65 (1.97)	63 (1.91)	54 (1.64)	62 (1.88)	90 (2.73)
KPC (+)	2	0	0	0	0	0	0	1	0	1	0	0
<i>Pseudomonas aeruginosa</i>	332	10 (1.0)	41 (4.1)	40 (4.0)	45 (4.5)	33 (3.3)	34 (3.4)	17 (1.7)	25 (2.5)	25 (2.5)	37 (3.7)	25 (2.5)
MBL (+)	11	0	0	0	0	0	0	0	2	2	6	1
MBL (–)	321	10 (1.0)	41 (4.1)	40 (4.0)	45 (4.5)	33 (3.3)	34 (3.4)	17 (1.7)	23 (2.3)	23 (2.3)	31 (3.1)	24 (2.4)
<i>Acinetobacter baumannii</i>	764	27 (1.0)	51 (1.89)	82 (3.4)	70 (2.59)	68 (2.52)	64 (2.37)	101 (3.74)	93 (3.44)	75 (2.78)	68 (2.52)	65 (2.41)
MBL (+)	2	0	0	0	0	0	0	2	0	0	0	0
MBL (–)	762	27 (1.0)	51 (1.89)	82 (3.4)	70 (2.59)	68 (2.52)	64 (2.37)	99 (3.67)	93 (3.44)	75 (2.78)	68 (2.52)	65 (2.41)
<i>Stenotrophomonas maltophilia</i>	96	2 (1.0)	3 (1.5)	0	12 (6.0)	16 (8.0)	17 (8.5)	10 (5.0)	1 (0.5)	9 (4.5)	12 (6.0)	14 (7.0)

Table 4 Drug susceptibility of non-fermenting Gram-negative bacilli in the period 2005–2015

Chemotherapeutics	<i>Acinetobacter baumannii</i>		<i>Pseudomonas aeruginosa</i>		<i>Stenotrophomonas maltophilia</i>	
	Susceptibility (%)	Resistance (%)	Susceptibility (%)	Resistance (%)	Susceptibility (%)	Resistance (%)
Ampicillin/Sulbactam	65	35	–	–	–	–
Cefepime	–	–	68	32	–	–
Ceftazidime	–	–	63	37	–	–
Colistin	100	0	100	0	–	–
Gentamicin	21	79	61	39	–	–
Imipenem	35	65	55	45	–	–
Meropenem	38	62	58	42	–	–
Piperacillin/Tazobactam	–	–	76	24	–	–
Trimethoprim/Sulfamethoxazole	–	–	–	–	100	0

Table 5 Drug susceptibility of Gram-negative bacilli of the Enterobacteriaceae family in the period 2005–2015

Chemotherapeutics	Enterobacteriaceae ESBL (+)		Enterobacteriaceae ESBL (–)		<i>Klebsiella pneumoniae</i> KPC (+)	
	Susceptibility (%)	Resistance (%)	Susceptibility (%)	Resistance (%)	Susceptibility (%)	Resistance (%)
Amikacin	42	58	95	5	50	50
Cefepime	0	100	91	9	0	100
Cefotaxime	0	100	88	12	0	100
Ceftazidime	8	92	87	13	0	100
Cefuroxime	0	100	70	30	0	100
Ciprofloxacin	14	86	82	18	0	100
Colistin ^a	100	0	100	0	100	0
Gentamicin	19	81	91	9	100	0
Imipenem	100	0	97	3	0	100
Meropenem	100	0	99	1	0	100
Piperacillin/Tazobactam	21	79	84	16	0	100
Tigecycline	52	48	76	24	100	0
Trimethoprim/Sulfamethoxazole	26	74	83	17	0	100

^a*Proteus mirabilis* and *Serratia marcescens* strains, naturally resistant to colistin were omitted

4 Discussion

There is a great likelihood that Gram-negative bacteria will no longer be susceptible to any of the available antibiotics; such cases have already been sporadically reported. Multidimensional infection control as outstandingly described for prevention of ventilator-assisted

pneumonia has thus become essential (Rosenthal et al. 2012). By following the recommended guidelines for pneumonia prevention, the prevalence of VAP can be reduced by up to 45 % (Resar et al. 2005). Di Pasquale et al. (2014) have reported 285 cases of VAP and 135 cases of non-VAP among 343 consecutive patients treated in ICU. The etiological examination performed in 63 % of cases demonstrated

that Gram-negative bacteria were responsible for 72.8 % of the infections, of which 41 % were caused by Enterobacteriaceae, including 29.2 % ESBL-producing strains. The present study, which was conducted among 2330 mechanically ventilated ICU patients, found that the majority of infections were associated with Enterobacteriaceae (42.0 %), including 10.9 % ESBL-producing strains. A study by Wojkowska-Mach et al. (2009) involving 2170 patients after cardiac surgery, 2.2 % of whom were diagnosed with VAP, has demonstrated that the most common pathogens isolated were *Klebsiella pneumoniae* (16.7 %), *Escherichia coli* (12.6 %), and *Pseudomonas aeruginosa* (10.4 %). Another study of ICU patients has found that the prevalence of ESBL-producing *Klebsiella pneumoniae* was 64.1 % in biological material from different sources, including 48.7 % bronchoalveolar lavage samples (Sękowska et al. 2014). Zeliaś et al. (2009) have studied 312 ICU patients, 40 of whom were diagnosed with VAP and found that Gram-negative bacteria were responsible for 71 % of infections, while *Escherichia coli*, *Klebsiella pneumoniae*, and ESBL-producing *Proteus mirabilis* for 10 % of infections. A large multi-center study involving 2436 patients from 27 different ICUs across Western Europe has found that 1089 of them were diagnosed with pneumonia, with the etiological factors of the following order: Enterobacteriaceae (43.8 %), *Pseudomonas aeruginosa* (23.1 %), and *Acinetobacter baumannii* (19.1 %) (Koulenti et al. 2009). In recent years, *Acinetobacter baumannii* has become an increasingly common cause of pneumonia carrying high mortality rates. It is a multi-drug resistant pathogen which is prevalent worldwide and has been isolated from soldiers participating in military operations conducted in Iraq and Afghanistan (Dallo and Weitao 2010).

Resistance of gram-negative bacteria to antibiotics varies between geographical areas. The most favorable epidemiological situation is reported from northern European countries and the least favorable from the southern European countries. A study by Hanberger et al. (2009) investigating antimicrobial resistance in ICUs

across Europe has shown that the resistance of *Acinetobacter baumannii* to imipenem and aminoglycosides is nil in Sweden, 4.8 % and 23 %, respectively, in the Czech Republic, 38.5 % and 80.2 % in Turkey, whereas it is as high as 90.9 % and 93.2 %, respectively, in Malta. The lowest resistance of *Pseudomonas aeruginosa* to ceftazidime was reported from Estonia (5.5 %) and the highest from Turkey (48.3 %) (Hanberger et al. 2009).

Another study investigating 23,918 Gram-negative bacterial strains sampled from ICU patients in six regions of the world between 2004 and 2009 has shown the lowest prevalence of *Klebsiella pneumoniae* and ESBL-producing *Escherichia coli* strains in North America (12.7 % and 4.7 %, respectively), and ESBL-producing strains have been reported from Latin America (45.5 %) and Africa (54.9 %). The susceptibility of *Acinetobacter baumannii* strains to meropenem varied across different regions, ranging from 60.4 % in North America to 15.9 % in Africa; the susceptibility of *Pseudomonas aeruginosa* to the above antibiotic ranged from 79.1 % in South America to 51.4 % in Africa (Bertrand and Dowzicky 2012). In all regions, ESBL-producing strains were found susceptible to imipenem; their susceptibility reaching up to 90 % (Hanberger et al. 2009). To-date, only have few studies investigated the prevalence and susceptibility of *Stenotrophomonas maltophilia*. In Poland, information on *Stenotrophomonas maltophilia*-associated infections can only be found in a study by Zeliaś et al. (2009) that reports a 3 % prevalence of this infection. In the present study, *Stenotrophomonas maltophilia* strains accounted for 5 % of all Gram-negative bacteria responsible for pneumonia. It is worth pointing out that we observed a considerable increase in the number of *Stenotrophomonas maltophilia*-associated infections, reaching up to 750 %. The reason for this is quite obvious; the pathogen is naturally resistant to carbapenems (Hankiewicz-Ziołkowska and Gospodarek 2014; Brooke 2012); the antibiotics that have become more commonly used for the management of pneumonia in recent years. Our present findings are similar to those reported by a majority of

other researchers as regards the susceptibility of ESBL-producing strains to imipenem and meropenem (Guzek et al. 2013; Kozioł-Montewka et al. 2011) and the susceptibility of KPC-producing strains to colistin and tigecycline and to a lesser degree to gentamycin (Robilotti and Deresinski 2014). Yet the findings are at variance with those in the literature as regards *Acinetobacter baumannii* and its susceptibility to carbapenems; 35 % in the present study vs. 70 % in other studies (Hankiewicz-Ziołkowska and Gospodarek 2014; Fishbain and Peleg 2010).

To improve the effectiveness of antibiotics against resistant strains, chemotherapeutics, especially the β -lactam group, should be administered by a continuous or extended infusion. In the treatment of *Acinetobacter baumannii*-associated infections, colistin should be administered together with other chemotherapeutics, such as rifampicin or minocycline, belonging to a group of tetracycline antibiotics. Infections caused by KPC-producing strains should be managed with a combination therapy including two antibiotics, providing the strains are susceptible (Watkins and Deresinski 2015).

In the present study, 98 % of biological samples were collected *via* a catheter inserted into an endotracheal or tracheostomic tube. The method is widely used due to its simplicity. In a study of 750 patients with suspected pneumonia, hospitalized in 28 different ICUs, there have been no significant differences between bronchoalveolar lavage and bronchial aspirate samples as regards the 28-day mortality and the type of antibiotics used (Canadian Critical Care Trials Group 2006). A study by Michel et al. (2005), comparing the quality of samples from both sources, have shown that the same strains are present in 89 % of specimens, regardless of the collection method, and that the choice of antibiotic therapy is correct in 95 % of cases in both groups of samples. Likewise, in a Polish study, the results of bacteriological tests have been similar for different methods of sample collection; be it bronchial aspirate, bronchoalveolar lavage, or protected specimen brush (Kowalczyk et al. 2011).

5 Conclusions

This 11-year long bacteriological study confirmed a trend which has been observed globally, i.e., a significant increase in the prevalence of pneumonia caused by Gram-negative bacteria in mechanically ventilated patients hospitalized in intensive care units. The greatest increase in the prevalence was observed for ESBL-producing *Enterobacteriaceae* and *Stenotrophomonas maltophilia*. Colistin remains the most effective drug against the majority of Gram-negative bacteria. Therapeutic problems commonly arise in the course of treatment of *Acinetobacter baumannii* infections.

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

References

- Bertrand X, Dowzicky MJ (2012) Antimicrobial susceptibility among gram-negative isolates collected from intensive care units in North America, Europe, the Asia-Pacific Rim, Latin America, the Middle East, and Africa between 2004 and 2009 as part of the Tigecycline Evaluation and Surveillance Trial. *Clin Ther* 34:124–137
- Brooke JS (2012) *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. *Clin Microbiol Rev* 25(1):2–41
- Canadian Critical Care Trials Group (2006) A randomized trial of diagnostic techniques for ventilator-associated pneumonia. *N Engl J Med* 355:2619–2630
- Coque TM, Baquero F, Canton R (2008) Increasing prevalence of ESBL-producing *Enterobacteriaceae* in Europe. *Euro Surveill* 13(47):pii:19044
- Dallo SF, Weitao T (2010) Insights into *Acinetobacter* war-wound infections, biofilms, and control. *Adv Skin Wound Care* 23(4):169–174
- Di Pasquale M, Ferrer M, Esperatti M, Crisafulli E, Giunta V, Li Bassi G et al (2014) Assessment of severity of ICU-acquired pneumonia and association with etiology. *Crit Care Med* 42(2):303–312
- Fishbain J, Peleg AY (2010) Treatment of *Acinetobacter* infections. *Clin Infect Dis* 51(1):79–84
- Guzek A, Tomaszewski D, Rybicki Z, Truszczyński A, Barański M, Korzeniewski K (2013) Comparison of in vitro efficacy of eropenem, imipenem and meropenem in the infections caused by the *Enterobacteriaceae* strains family. *Anaesthesiol Intensive Ther* 45:69–79

- Hanberger H, Arman D, Gill H, Jindrak V, Kalenic S, Kurcz A et al (2009) Surveillance of microbial resistance in European Intensive Care Units: a first report from the Care-ICU programme for improved infection control. *Intensive Care Med* 35(1):91–100
- Hankiewicz-Ziołkowska H, Gospodarek E (2014) Resistance mechanisms to antibiotics and chemotherapeutics in *Stenotrophomonas maltophilia*. *Post Mikrobiol* 53(2):135–140 (Article in Polish)
- Kollef MH, Shorr A, Tabak YP, Gupta V, Liu LZ, Johannes RS (2005) Epidemiology and outcomes of health-care-associated pneumonia: results from a large US database of culture-positive pneumonia. *Chest* 128(6):3854–3862
- Koulenti D, Lisboa T, Brun-Buisson C, Krueger W, Macor A, Sole-Violan J et al (2009) Spectrum of practice in the diagnosis of nosocomial pneumonia in patients requiring mechanical ventilation in European intensive care units. *Crit Care Med* 37(8):2360–2368
- Kowalczyk W, Rybicki Z, Tomaszewski D, Truszczyński A, Guzek A (2011) The comparison of different bronchial aspirate culturing methods in patients with ventilator-associated pneumonia (VAP). *Anaesthesiol Intensive Ther* 43(2):74–79 (Article in Polish)
- Kozioł-Montewka M, Jaworska-Gromaszek I, Biernacka J, Pluta A, Niedźwiadek J, Kaczor D et al (2011) Review of the effectiveness of an empirical antibiotic therapy in suspected ventilator-associated pneumonia. *Anaesthesiol Intensive Ther* 43(3):163–168 (Article in Polish)
- Michel F, Franceschini B, Berger P, Amal JM, Gannier M, Sainty JM, Papazian L (2005) Early antibiotic treatment for BAL-confirmed ventilator-associated pneumonia: a role for routine endotracheal aspirate cultures. *Chest* 127(2):589–597
- Resar R, Pronovost P, Haraden C, Simmonds T, Rainey T, Nolan T (2005) Using a bundle approach to improve ventilator care processes and reduce ventilator-associated pneumonia. *Jt Comm J Qual Patient Saf* 31(5):243–248
- Robilotti E, Deresinski S (2014) Carbapenemase-producing *Klebsiella pneumoniae*. *F1000Prime Rev* 6:80; doi: 10.12703/P6-80
- Rosenthal VD, Rodrigues C, Alvarez-Mureno C, Madania N, Mitrev Z, Ye G, INICC members et al (2012) Effectiveness of a multidimensional approach for prevention of ventilator-associated pneumonia in adult intensive care units from 14 developing countries of four continents: findings of the International Nosocomial Infection Control Consortium. *Crit Care Med* 40(12):3121–3128
- Sękowska A, Gospodarek E, Kusza K (2014) The prevalence of infections and colonisation with *Klebsiella pneumoniae* strains isolated in ICU patients. *Anaesthesiol Intensive Ther* 46(4):295–298
- Torres A, Ewig S, Lode H, Carlet J, European HAP Working Group (2009) Defining, treating and preventing hospital acquired pneumonia: European perspective. *Intensive Care Med* 35(1):9–29
- Vincent JL, Bihari DJ, Suter PM, Bruining HA, White J, Nicolas-Chanoin MH et al (1995) The prevalence of nosocomial infection in intensive care units in Europe. Results of the European Prevalence of Infection in Intensive Care (EPIC) study. *JAMA* 274(8):639–644
- Vincent JL, Rello J, Marshall J, Silva E, Anzueto A, Martin CD et al (2009) International study of the prevalence and outcomes of infection in intensive care units. *JAMA* 302(21):2323–2329
- Watkins RR, Deresinski S (2015) Is combination therapy for carbapenem-resistant *Klebsiella pneumoniae* the new standard of care? *Expert Rev Anti Infect Ther* 13(4):405–407
- Wojkowska-Mach J, Baran M, Drwila R, Ziętkiewicz M, Foryciarz E, Synowiec E et al (2009) Ventilator-associated pneumonia after procedures in cardiac surgery. *Anaesthesiol Intensive Ther* 41(4):224–229 (Article in Polish)
- Zeliaś A, Budak A, Włodarczyk D, Wodziński P (2009) Quantitative culture sampling of tracheal aspirates for diagnosis of nosocomial pneumonia in the ITU. *Anaesthesiol Intensive Ther* 41(2):100–104 (Article in Polish)

Whooping Cough in Adults: A Series of Severe Cases

K. Zycinska, M. Cieplak, M. Chmielewska, A. Nitsch-Osuch, A. Klaczkow, M. Hadzik-Blaszczyk, Z. Kur, and K. A. Wardyn

Abstract

Bordetella pertussis is a gram-negative aerobic coccobacillus causing contagious respiratory tract disease called whooping cough. The virulence factors consist of pertussis toxin, filamentous hemagglutinin, fimbriae, lipooligosaccharide, and adenylate cyclase toxin. The disease causes a worldwide threat to public health despite a high vaccination coverage. The course of whooping cough in adults is frequently atypical, causing difficulty in diagnosis. In this report we present five patients hospitalized with *Bordetella pertussis* infection manifesting atypical and severe symptoms. The diagnosis was based on serological tests: serum concentration of specific antibodies against pertussis toxin and sputum cultures. We observed a wide spectrum of symptoms, from benign (sinus pain – 80 %, headaches – 20 %), through moderate (hemoptysis – 40 %; chest pain 60 %) to severe symptoms (cardiac arrhythmia – 40 %; syncope – 60 %). *Bordetella pertussis* infection can cause life-threatening complications and exacerbation of concomitant chronic diseases. Most vaccination programs cover only the first few months of life. Booster doses should be considered in adults, especially those immunocompromised or with pulmonary complications, but also in healthcare workers who are exposed to the contagion and also may spread the infection.

Keywords

Bordetella pertussis • Cardiac arrhythmia • Chronic cough • Syncope • Vaccination booster • Whooping cough

K. Zycinska (✉), M. Cieplak, M. Chmielewska, A. Nitsch-Osuch, A. Klaczkow, M. Hadzik-Blaszczyk, Z. Kur, and K.A. Wardyn
Department of Family Medicine with Internal and Metabolic Diseases Ward, Warsaw Medical University, 19/25 Stępińska Street, 00-739 Warsaw, Poland
e-mail: kzycinska@poczta.fm

1 Introduction

Whooping cough is an infection of the respiratory tract caused by *Bordetella pertussis*, a gram-negative aerobic coccobacillus. The virulence

factors consist of pertussis toxin, filamentous hemagglutinin, fimbriae, adenylate cyclase toxin, lipooligosaccharide, pertactin, and tracheal cytotoxin. These factors enable the bacteria to adhere to the cytoskeleton of the ciliated cells of the airway epithelium, causing local inflammation and impairment of secretion clearing (Scheller and Cotter 2015; Scheller et al. 2015). As a result the most characteristic symptom of whooping cough arises; paroxysmal and unproductive cough, followed by a high-pitched whoop. Typically, the incubation period of the infection is 7–14 days, but it ranges from 4 to up to 21 days (Centers for Disease Control and Prevention 2012). The disease is highly communicable with a transmission rate of 80 %. The infected people are most contagious for up to two weeks after onset of symptoms. The natural course of pertussis infection can be divided in three stages. The first one, lasting for two weeks, is a catarrhal stage presenting symptoms of upper respiratory tract inflammation, which are similar to those of common cold, such as rhinorrhea, sneezing, headache, mild fever, and sore throat. Then, a paroxysmal stage develops, with unproductive cough, often accompanied by choking and vomiting, which leaves the patient exhausted. Symptoms become routinely aggravated in the evening or at night. During the attack, which can last up to 5 min, cyanosis can occur. During the following 2–3 weeks, frequency of attacks decreases, evolving into the convalescent stage with a slow regression of symptoms. The course of disease is frequently atypical in adolescents and adults. Cough may not be characteristic, especially in those previously vaccinated. Nonetheless, disease may be life-threatening not only for infants. In the adult population, often burdened with concomitant chronic diseases, a severe course of pertussis infection can cause such symptoms as cardiac arrhythmias, syncope, hypoxia, which can be life-threatening (Cornia et al. 2010). In this report we demonstrate a series of atypical, difficult to diagnose cases of *Bordetella pertussis* infection in adult patients, having a potentially life-threatening course.

2 Methods

The study was approved by a local Ethics Committee of the Medical University of Warsaw, Poland. We describe nine cases of *Bordetella pertussis* infection in adult patients, five women aged 40–69 and four men aged 45–60, hospitalized in the ward of Family Medicine, Internal, Metabolic Diseases of the Medical University of Warsaw between January 2014 and September 2015. Pertussis infection is rather uncommon in patients admitted to internal medicine wards. The patients were admitted due to atypical symptoms raising diagnostic issues and necessitating to broaden diagnostic procedures. The diagnostics was mainly based on serological tests. Sputum culture on Bordet-Gengou agar plates specific for *Bordetella pertussis* was negative in 7 patients. We performed ELISA test for immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) specific for *Bordetella pertussis*. In five patients, IgA was positive, ranging from 12.0 to 25.3 NTU (number of transfer units) and IgG was positive in six patients, ranging from 14.5 to 27.6 NTU. In one patient, IgM was only positive, with a concentration of 14.7 NTU. We opted for serological testing to confirm the diagnosis rather than PCR or direct fluorescent antibody staining of nasopharyngeal secretions due possibly to a lower effectiveness of those other methods in case of longer lasting infections (Bock et al. 2012; Sanz Moreno et al. 2002). The average time from onset of symptoms to diagnosis in our patients was 29 (range 25–35) days. The polymerase chain reaction (PCR) conducted on sputum samples is recently considered the most effective method of diagnosis (Leber 2014), and a study by Stone et al. (2014) has suggested the PCR effectiveness can last up to 110 days of onset of *Bordetella pertussis* disease. The long lasting PCR diagnostic effectiveness, however, does not seem to have been fully verified. All patients described herein had chest X-rays performed, which showed no specific abnormalities, and had laryngological, due to protracted cough, and neurological, due to syncope episodes, examinations.

3 Results and Discussion

The patients described in the present report underwent vaccination in childhood according to the past immunization schedules that failed to recommend booster doses for adults. They manifested a wide range of symptoms; mild caused by the inflammatory process in the upper airways causing cough persisting for more than 3 weeks, hoarseness, sinus pains, headaches, and influenza-like symptoms with muscle and joint soreness mostly located in the chest, spine, and shoulders, and weight loss and sleep disturbances. Those symptoms persisted throughout the infection and affected the patients' everyday life routine. In addition, some of the patients manifested symptoms which were directly associated with the paroxysmal cough with the accompanying inspiratory whoop such as chest pain, hemoptysis, and urinary incontinence. The most severe and dangerous symptoms which made the patients seek help in the emergency unit and caused hospitalization were posttussive vomiting or choking, seizures, cardiac arrhythmia, and syncope. The incidence of most common symptoms of *Bordetella pertussis* infection observed in the study is depicted in Table 1.

Table 1 Incidence of most common symptoms of *Bordetella pertussis* infection in a group of nine patients

Symptom	No. of patients
Posttussive vomiting or choking	6
Urinary incontinence	3
Sleep disturbance	6
Sweating attack	3
Weight loss	2
Seizure	4
Syncope	4
Chest pain	7
Hemoptysis	3
Headache	3
Hoarseness	5
Sneezing attacks	4
Influenza-like symptoms	8
Sinus pain	8
Cardiac arrhythmia	6
Chronic cough	9
Cyanosis	3

Typical treatment consisted of macrolides or cotrimoxazole, the combination of trimethoprim (TMP) and sulfamethoxazole (SMX), in those with contradictions for the use of macrolides. After receiving the antimicrobial treatment, the patients presented in this report slowly recovered; with the cough attacks ceasing within few days. The following antibiotics could be used: azithromycin 500 mg orally as a single dose on the first day followed by 250 mg orally once a day on days 2 through 5, clarithromycin for 7 days in a dose of 1 g/24 h, erythromycin for 14 days in a dose of 2 g/24 h, and cotrimoxazol (TMP/SMX – 160/800 mg twice daily).

Whooping cough commonly involves children but is also present in the adult population. The incidence decreases in older age groups, from app. 17/100,000 in young adults to 5/100,000 in persons aged over 65. There were more than 38,200 confirmed cases of whooping cough in the EU in 2012. The incidence rate almost quadrupled in a three-year period between 2012 and 2010 (Table 2) (ECDC 2014). A concern has lately arisen about a global resurgence of pertussis due to the observable increase in the incidence. However, a growing number of pertussis cases noted over several recent years is mainly attributed to the natural epidemic cyclic pattern, occurring every 2–5 years (Bhatti et al. 2015). Nonetheless, there is evidence that a genuine resurgence has occurred in Australia, Chile, Portugal, UK, USA, where acellular vaccine (aP) vaccines were exclusively used (WHO

Table 2 Confirmed *Bordetella pertussis* cases

Country	2012	2011	2010
Poland	4684	678	573
United Kingdom	11,993	1256	366
Portugal	237	32	13
Italy	262	516	463
Spain	1804	1013	305
France	198	71	50
Austria	571	109	236
Norway	4247	4368	3560
Greece	56	2	55
EU total	38,242	12,700	10,777

Data from annual epidemiological report on vaccine-preventable diseases, ECDC Surveillance Report 2014

2015). It seems that whooping cough still persists even in high income countries despite the primary prevention. To-date two types of pertussis vaccines are available: whole-cell (wP) vaccine based on attenuated *Bordetella pertussis* organisms, and aP vaccine based on highly purified individual pertussis antigens. A primary course of three doses of vaccine, usually combined with diphtheria and tetanus, is usually given between 2 and 12 months of age. Booster doses are recommended at 11–24 months of age and between 3 and 6 years of age. There is a considerable variation between national immunization schedules in administering the vaccine. Some countries, including Poland beginning as of 2015, recommend boosters for adolescents and during pregnancy (optimally in the third trimester) or soon after delivery (Centers for Disease Control and Prevention 2015).

Whooping cough still poses a worldwide threat to the public health despite high vaccination coverage which globally reached 86 % in 2014 (WHO 2015). The course of whooping cough in adults is frequently atypical due to previous antibiotic treatment, vaccinations, and the use of over-the-counter medicines, all of which causes diagnostic problems. The infection, usually rather mild in older population, can cause life-threatening complications and exacerbations of concomitant chronic diseases, which often results in patient hospitalization. Pertussis infection should be taken into consideration in differential diagnosis of chronic cough. It is potentially a preventable disease due to the existing vaccination programs. Patients, in particular pregnant women, immunocompromised, and those with chronic comorbidities, should be encouraged to take booster doses. Interestingly, among the patients of the present study there were two physicians, two nurses, and a kindergarten caregiver. Thus, it seems reasonable to administer a booster dose as a preventive measure to healthcare workers and those working with children, who are susceptible not only to acquire but also to spread the infection.

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

References

- Bhatti MM, Rucinski SL, Schwab JJ, Cole NC, Gebrehiwot SA, Patel R (2015) Eight-year review of *Bordetella pertussis* testing reveals seasonal pattern in the United States. *J Pediatric Infect Dis Soc* pii:piv079
- Bock JM, Burtis CC, Poetker DM, Blumin JH, Frank MO (2012) Serum immunoglobulin G analysis to establish a delayed diagnosis of chronic cough due to *Bordetella pertussis*. *Otolaryngol Head Neck Surg* 146:63–67
- Centers for Disease Control and Prevention (2012) Pertussis epidemiology and prevention of vaccine-preventable diseases, (12 ed). Atkinson W, Wolfe S, Hamborsky J, eds. Washington, DC: Public Health Foundation, pp 215–230
- Centers for Disease Control and Prevention (2015) The pink book: course textbook. Epidemiology and prevention of vaccine-preventable diseases (13 ed) U.S. Department of Health and Human Services, pp 261–278
- Cornia PB, Hersh AL, Lipsky BA, Newman TB, Gonzales R (2010) Does this coughing adolescent or adult patient have pertussis? *JAMA* 304:890–896
- ECDC (2014) European Centre for Disease Prevention and Control. Annual epidemiological report. Vaccine-preventable diseases, ECDC Surveillance Report. Available from: <http://www.ecdc.europa.eu>; Accessed on 1 Sept 2015
- Leber AL (2014) Pertussis: relevant species and diagnostic update. *Clin Lab Med* 34:237–255
- Sanz Moreno JC, De Ory Manchón F, González Alonso J, de La Torre JL, Salmerón F, Limia A, Tello O, Pachón I, Amela C, Vázquez J, de Ory F, Sanz JC; Grupo de Trabajo sobre Tos Ferina (2002) Laboratory diagnosis of pertussis. Role of serology. *Enferm Infecc Microbiol Clin* 20:212–218 (Article in Spanish)
- Scheller EV, Cotter PA (2015) *Bordetella* filamentous hemagglutinin and fimbriae: critical adhesins with unrealized vaccine potential. *Pathog Dis* 73(8): ftv079; doi: [10.1093/femspd/ftv079](https://doi.org/10.1093/femspd/ftv079)
- Scheller EV, Melvin JA, Sheets AJ, Cotter PA (2015) Cooperative roles for fimbria and filamentous hemagglutinin in *Bordetella* adherence and immune modulation. *Mbio* 6(3):e00500-15. doi:[10.1128/mBio.00500-15](https://doi.org/10.1128/mBio.00500-15)
- Stone BL, Daly J, Srivastava R (2014) Duration of *Bordetella pertussis* polymerase chain reaction positivity in confirmed pertussis illness. *J Pediatric Infect Dis Soc* 3:347–349
- WHO (2015) Pertussis vaccines: WHO position paper. The Weekly Epidemiological Record (WER) No. 35 2015, 90(35):433–460 Available from: <http://www.who.int/wer>; Accessed on 15 Aug 2015

Limited Clinical Significance of Dimeric Form of Pyruvate Kinase as a Diagnostic and Prognostic Biomarker in Non-small Cell Lung Cancer

Adam Rzechonek, Aleksandra Kaminska, Piotr Mamczur, Arkadiusz Drapiewski, and Władysław Budzynski

Abstract

Metabolism of tumor tissue differs from the normal one by the intensity of protein synthesis and glycolysis. The dimeric pyruvate kinase (PKM2) is a specific enzyme for tumor glycolysis. The aim of this study was to determine the relationship between the activity of PKM2 and the type and stage of non-small cell lung cancer (NSCLC). A second objective was to compare the expression of PKM2 with disease progression and prognosis. We studied 65 patients divided into two groups: 45 patients with lung cancer and 20 non-cancer healthy subjects taken as control. The serum activity of PKM2 was assessed spectrophotometrically. We found that PKM2 activity was greater, on average, by 136 % for adenocarcinoma and for 126 % for squamous cell carcinoma compared with that present in control subjects. The higher PKM2 activity was associated only with Stage III of cancer ($p < 0.001$). Sensitivity of PKM2 as a cancer marker was 79 % for adenocarcinoma and 81 % for squamous cell carcinoma and specificity was 50 % for both cancer types. We conclude that PKM2 activity is higher in patients with NSCLC than in healthy subjects. The level of PKM2 activity is associated with advanced stage of cancer. Nonetheless, low specificity of PKM2 assessment makes it of limited utility in NSCLC diagnosis or evaluation of cancer progression.

A. Rzechonek (✉)
Department of Thoracic Surgery, Wrocław Medical
University, 105 Grabiszynska St., 53-439 Wrocław,
Poland

Lower Silesian Center for Lung Diseases, Wrocław,
Poland
e-mail: adam.rzechonek@gmail.com

A. Kaminska and A. Drapiewski
Department of Thoracic Surgery, Wrocław Medical
University, 105 Grabiszynska St., 53-439 Wrocław,
Poland

P. Mamczur
Department of Animal Physiology, Faculty of Biological
Sciences, Wrocław University, 30 Cybulskiego St.,
Wrocław, Poland

W. Budzynski
Biotech Consultant, 56 Pilsudskiego Street,
50-033 Wrocław, Poland

Keywords

Biomarker • Cancer tissue • Cancer stage • Disease progression • Glycolysis • Lung cancer • Prognosis • Pyruvate kinase • Sensitivity • Specificity • Non-small cell lung cancer

1 Introduction

In cancer tissue, an increase of glucose consumption is observed, along with enhanced anaerobic transformation - glycolysis. Cancer cells take up much larger quantities of glucose than normal cells and they exhibit a high activity of glycolysis and lactate formation. The increase of anaerobic reactions in cancer cells, called the Warburg Phenomenon (Warburg 1956), may be due to hypoxia occurring in ischemic regions of tumor tissue. Anaerobic reactions compensate for tissue hypoxia facilitating survival of cancer cells. The key metabolite of this biochemical transformation is pyruvate kinase, an enzyme involved in the breakdown of glucose, whose action leads to the formation of pyruvate and ATP. A metabolically active tetrameric isoform of pyruvate kinase is tissue-specific for healthy organs. The presence of a dimeric form of kinase (PKM2) in the serum, devoid of catalytic properties, points to an ongoing cancerous process. The PKM2 is associated with the ability of cancer cells to synthesize proteins, nucleic acids, and lipids. This enables cell division, and tumor growth and aggressiveness (Roudier and Perrin 2009). In addition, increased PKM2 concentration facilitates efficient synthesis of ATP under oxygen deficiency and protects cancer cells from apoptosis (Kobierzycki et al. 2014; Christofk et al. 2008).

Among the non-small cell lung cancers (NSCLC), adenocarcinomas constitute the most aggressive group. Squamous cell carcinoma has a lower aggressiveness; it slower metastasizes and causes a relatively longer survival. The PKM2 occurs in both these types of NSCLC. The association of PKM2 expression in lung cancer tissue with the main pathological characteristics of cancer has been previously

shown as rather irrelevant (Voorzanger-Rousselot and Garnerio 2007). However, studies on the clinical importance of serum PKM2 in NSCLC are scant and the issue is unsettled. Therefore, the aim of the present study was to evaluate PKM2 activity in patients diagnosed with squamous cell carcinoma or adenocarcinoma in the lung and to seek the possible association of PKM2 with tumor progression and prognosis.

2 Methods

The study was approved by the Bioethics Committee of Wrocław Medical University in Wrocław, Poland, and was conducted in accord with the principles set by the declaration of Helsinki for Human Research.

Sera of 65 individuals were tested. There were 45 patients diagnosed with non-small cell lung cancer (F/M – 17/28, median age – 67, range 42–83 years). Adenocarcinoma was diagnosed in 24 and squamous cell carcinoma in 21 patients. Patients were classified according to stage grouping of the TNM subsets (T = primary tumor, N = regional lymph nodes, M = distant metastasis) in the recently revised International System for Staging Lung Cancer (Mirsadraee et al. 2012; Mountain 1997). The patients diagnosed with adenocarcinoma were operated in Stages IIB to IIIA, and those with squamous cell carcinoma underwent surgery in Stage IIA to IIIA (Table 1). The control group consisted of 20 healthy volunteers (F/M – 10/10; median age 53.5, range 22–76 years).

All patients had a histopathology diagnosis at the time of surgery. The PKM2 was assessed using a spectrophotometric method in samples of blood serum taken just before surgery. A

Table 1 Basic demographics of patients with non-small cell lung cancer (NSCLC), cancer type and stage, and increases in serum pyruvate kinase (PKM2) activity in response to the added biochemical activators

Gender	Age (years)	Cancer stage	Histopathological diagnosis	% increase in PKM2
M	42	II A	Adenocarcinoma	104
M	44	III A	Adenocarcinoma	106
M	78	I B	Squamous lung cancer	106
M	83	III A	Squamous lung cancer	106
F	54	I B	Squamous lung cancer	106
F	56	II A	Squamous lung cancer	108
F	48	III A	Adenocarcinoma	112
M	66	II A	Adenocarcinoma	113
M	48	II A	Adenocarcinoma	114
M	68	II	Adenocarcinoma	118
F	64	I A	Squamous lung cancer	118
M	57	II A	Adenocarcinoma	119
F	56	II A	Adenocarcinoma	119
M	78	I B	Squamous lung cancer	122
M	67	III A	Adenocarcinoma	123
F	67	II A	Adenocarcinoma	123
F	70	I B	Squamous lung cancer	123
F	80	II A	Squamous lung cancer	126
M	56	II A	Adenocarcinoma	127
M	60	I B	Squamous lung cancer	127
M	55	I B	Squamous lung cancer	128
F	78	II A	Squamous lung cancer	129
F	70	II A	Adenocarcinoma	130
M	42	III A	Adenocarcinoma	130
M	81	I B	Adenocarcinoma	132
M	80	II A	Adenocarcinoma	132
M	66	I B	Squamous lung cancer	132
F	60	II A	Adenocarcinoma	135
M	58	II A	Adenocarcinoma	136
M	58	II A	Adenocarcinoma	137
F	72	I A	Squamous lung cancer	140
M	52	II A	Adenocarcinoma	142
F	71	III A	Squamous lung cancer	143
M	78	III A	Squamous lung cancer	149
F	55	III A	Adenocarcinoma	154
F	78	III A	Squamous lung cancer	154
M	72	III A	Adenocarcinoma	156
F	67	III A	Squamous lung cancer	157
M	81	III A	Squamous lung cancer	158
F	59	II A	Adenocarcinoma	164
M	79	III A	Squamous lung cancer	165
M	78	III A	Adenocarcinoma	174
M	72	III A	Squamous lung cancer	174
M	68	III A	Adenocarcinoma	176
M	57	I B	Squamous lung cancer	214

difference in PKM2 activity was measured colorimetrically before and after the addition of the activators phosphoenolpyruvate and 1, 6-bisphosphate to the reaction mixture, using a Hewlett Packard 8900A spectrophotometer (Palo Alto, CA). The reaction measured was the rate of oxidation of NADH ($\lambda = 340$ nm). A unit of enzyme activity was determined as a quantity which catalyzes the transformation of 1 μ mol of substrate during 1 min at 37 °C, pH 7.5. The activity units and the percentage of increase was calculated in each group. Data are presented as means \pm SE and medians. Intergroup differences were statistically compared with one-way ANOVA. A p-value ≤ 0.05 was used to define statistically significant differences.

3 Results

In the control group, PKM2 activity increased in response to the activators by an average of 16.0 ± 2.8 % over the baseline level. This value was adopted as the threshold level for the assessment of sensitivity and specificity of PKM2 in lung cancer. In the lung cancer patients, adenocarcinoma and squamous cell carcinoma combined, PKM2 activity increased in individual sera by 4–114 %; the average increase amounted to 34.7 ± 3.5 (median 30)% over the baseline level, which was significantly higher than the 116 % increase in controls ($p = 0.001$). Concerning the specific type of NSCLC, average increase in PKM2 activity amounted to 32.3 ± 4.1 (median 30)% for adenocarcinoma and 37.4 ± 5.8 (median 29)% for squamous cell carcinoma ($p = 0.005$ for both compared with the baseline level in healthy subjects); the difference in the PKM2 increases between the two cancer types was insignificant.

PKM2 activity was increasing with tumor burden assessed according to the T subsets characteristics (T = primary tumor) of the International System for Staging Lung Cancer (Mirsadraee et al. 2012). The greater the tumor size the greater was the PKM2 activity, with a

significant difference between T1 and T2 subsets ($p = 0.019$) (Table 2).

We attempted to validate the potential accuracy of a biochemical test for PKM2 activity for diagnosing NSCLC as measured by sensitivity and specificity. The overall sensitivity, i.e., the ability of a test to correctly classify an individual as suffering from NSCLC, amounted to 64 %, being the highest for the T2 subset of cancer size according to the staging system. The overall specificity, i.e., the ability of a test to correctly identify a patient as NSCLC free amounted to 88 %, being the smallest for the T2 subset of cancer size (Table 3). Thus, sensitivity of PKM2 activity in the detection of NSCLC is rather low, with the possible exception of the T2 subset of patients where it becomes moderate. There were no appreciable differences in sensitivity/specificity of PKM2 test separately for adenocarcinoma and squamous cell carcinoma. However, a very limited number of cases of bigger tumors belonging to T3–T4 subsets makes the assessment of sensitivity dubious. During a 4-year follow-up period, nine NSCLC patients died as compared with one control subject who died of cancer unrelated reasons.

Table 2 Serum dimeric pyruvate kinase (PKM2) activity in relation to the T subsets (T = primary tumor) of the International System for Staging Lung Cancer

Feature T	No. of cases	PKM2 activity (%)
T1	16	21.3 ± 3.4
T2	25	$40.4 \pm 5.1^*$
T3	3	56.0 ± 4.7
T4	1	43.0

Enzyme activity is expressed as mean \pm SE percentage increase over the baseline level set for healthy control subjects; * $p = 0.019$ vs. T1

Table 3 Sensitivity and specificity of PKM2 activity assessment in relation to the T subsets (T = primary tumor) of the International System for Staging Lung Cancer

	T1	T2	T3	T4	Average
Sensitivity	50 %	84 %	0 %	0 %	64 %
Specificity	86 %	40 %	100 %	100 %	88 %

4 Discussion

A long-lasting search for a distinct and reliable biochemical marker of lung cancer has by far been unsuccessful (Voorzanger-Rousselot and Garnero 2007). In the present study we attempted to measure the serum activity of PKM2 in the hope that the level of this enzyme might have to do with the lung cancer development, and thus might be helpful in the diagnosis of disease and the prediction of its progression. We addressed the issue by investigating patients suffering from the two most common and aggressive, in terms of metastasizing, short survival, and treatment ineffectiveness, types of NSCLC, adenocarcinoma and squamous cell carcinoma. The findings demonstrate that PKM2 activity was indeed enhanced by about 30–40 % in both cancer types investigated, compared with healthy control subjects; the enhancement tended to be greater in more advanced cancer mass and stage. Nonetheless, enhancement of PKM2 activity, although significant, remained rather modest and erratic,

and its sensitivity in a range of 60 odd percentage points, in our opinion, seriously hampers the predicting power of PKM2 activity testing as a line of judgement on the NSCLC course. We confirmed the enhancement of PKM2 in NSCLC, reported in an earlier study by Papadaki et al. (2014), but in opposition to that study the present findings shed doubt on the argument that PKM2 expression may be a predictive biomarker of chemotherapy and thus the course of disease. A moderate diagnostic sensitivity of PKM2, regarding NSCLC, found in the present study is, generally, in line with those reported in a range of other studies that have assessed the PKM2 diagnostic value in different cancers; notably cancers of various organs of the digestive tract, where the sensitivity ranged between 47 and 68 %, with a couple of exceptions showing sensitivity above 90 %. A brief synopsis of the relevant literature is shown in Table 4.

Nonetheless, most authors of the reports collated in Table 4 have evaluated the clinical usefulness of PKM2 kinase assay as positive. None

Table 4 Literature excerpts concerning the assessment of a biomarker role of PKM2 in non-small cell lung cancer (NSCLC)

Article	No. of patients	Location of cancer	Material	Sensitivity	Predictive rating
Demir et al. (2013)	85	Colorectal	Serum	–	Positive
Li et al. (2012)	Review	Colorectal	Serum	77.0 %	Positive
Abdullah et al. (2012)	328	Colorectal	Serum	71.0 %	Positive
Zhang et al. (2004)	54	Gastric cancer	Serum	50.5 %	Positive
	54	Colorectal cancer		68.5 %	
Hardt and Ewald (2008)	Review	Gastrointestinal	Serum	55–75 %	Positive
		Colorectal	Feces	68.8–91.0 %	
Schulze (2000)	463	Gastrointestinal	Serum	89 % overall	Positive
				48–73 % location specific	
Schneider and Schulze (2003)	250	Colorectal	Serum	47.8 %	Positive
	122	Gastric		57.0 %	
	86	Esophageal		55.8 %	
Kumar et al. (2008)	50	Colorectal with meta ad liver	Serum	68.0 %	##
Kumar et al. (2007)	Meta-analysis	Pancreatic	Serum	95.0 %	Positive
Kobierzycki et al. (2014)	218	Lung	Tumor tissue	–	Negative
Garon et al. (2014)	Panel of 54 cell lines	Lung/Breast	Cell lines	–	Negative

PKM2 elevated, but no association with tumor volume, number of metastases or differentiation

of them, however, have pointed to the designation of PKM2 as sufficient to establish the diagnosis of cancer. On the other hand, sensitivity of serum PKM2 activity seems arguably greater than that of the reference tumor marker CEA or fecal occult blood testing in case of colorectal cancer (Abdullah et al. 2012; Hardt and Ewald 2008; Zhang et al. 2004; Hardt et al. 2003). The level of PKM2 activity is unassociated with the degree of differentiation and stage of cancer or with the number and size of metastases as described in a study on colorectal liver metastases (Kumar et al. 2008) in which the increase in PKM2 was diagnostically more telling than that in CEA. A somewhat more frequent increase in PKM2 than in CEA in cancer patients does not necessarily make the assessment of PKM2 activity superior, but rather points to the still unresolved search for good biomarkers in cancer diagnostics. In line with this reasoning, Ervens et al. (2008) have found that PKM2 cannot be considered a diagnostic marker in oral cancer due to low sensitivity and specificity of 63 % and 59 %, respectively, in stages T3 and T4 of this cancer type. Such a low level of sensitivity of PKM2 estimation is about the same as that found in the present study in the serum of NSCLC patients. We also failed to confirm the existence of an appreciable association of PKM2 with cancer mass, beginning cancer stages, or survival rate, although a small number of study patients hardly makes these assessments conclusive. Even if PKM2 activity increased in advanced stages of lung cancer, that would be of no clinical and practical meaning in view of the obvious diagnostic picture. Therefore, utility of PKM2 as a diagnostic or prognostic marker in lung cancer is highly questionable. Our present findings are at variance with those of Peng et al. (2011) who have demonstrated in a bigger cohort of patients that PKM2 activity correlates with poor prognosis in lung adenocarcinoma. PKM2 activity has also been found of prognostic significance in colon cancer, with the serum level elevated above 27 U/ml being associated with worse prognosis (Goonetilleke et al. 2007). In contrast, Garon et al. (2014) have reported that oral dichloroacetate, a compound that inhibits PKM2, has no beneficial effect on disease

progression in previously treated advanced stage IIIB/IV NSCLC patients. Putting aside inter-study differences, likely explicable by different study designs and different patient groups, PKM2 assessment may be of greater value when performed in combination with other standard markers, such as CEA. Intriguingly, Meng et al. (2015) have demonstrated that knockdown of enhanced PKM2 expression in NSCLC cell lines increases the radiosensitivity of cancer cells and xenografts. Likewise, Peng et al. (2011) have found that knockdown of PKM2 expression by RNA interference suppresses cell growth and induces apoptosis in a pulmonary adenocarcinoma cell line in vitro and suppresses tumor growth in a xenograft model in vivo. In line with those reports, Papadaki et al. (2014) have found that PKM2 expression negatively correlates with platinum sensitivity in advanced NSCLC. These findings open up a new avenue of usefulness of PKM2 activity in treatment rather than diagnosis of NSCLC.

In conclusion, we found that PKM2 activity is moderately enhanced in NSCLC patients compared with healthy subjects. However, we failed to demonstrate any clinically meaningful association between PKM2 and tumor mass, stage, or survival rate. Nor was PKM2 level of a help in differentiation between lung adenocarcinoma and squamous cell carcinoma. These findings, along with contentious literature data on a biomarker role of PKM2 in cancer, make us believe that PKM2 is of little, if any, clinical utility in lung cancer diagnosis and prognosis of its course. A possibly more distinct enhancement of PKM2 in terminally advanced cancer stages is of no influence on the opinion above expressed. A role of PKM2 inhibition in increasing therapeutic effects of radio- and chemosensitivity seems a viable area of further research, with a clear beneficial potential for cancer patients, and should be further pursued with alternative study designs.

Acknowledgements This work was supported by the statutory budget of Wrocław Medical University in Poland.

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

References

- Abdullah M, Rani AA, Simadibrata M, Fauzi A, Syam AF (2012) The value of fecal tumor M2 pyruvate kinase as a diagnostic tool for colorectal cancer screening. *Acta Med Indones* 44(2):94–99
- Christofk HR, Vander Heiden MG, Wu N, Asara JM, Cantley LC (2008) Pyruvate kinase M2 is a phosphotyrosine-binding protein. *Nature* 452 (7184):181–186
- Demir AS, Erdenen F, Müderrisoğlu C, Toros AB, Bektaş H, Gelışgen R, Tabak Ö, Altunoğlu E, Uzun H, Erdem Huq GE, Aral H (2013) Diagnostic and prognostic value of tumor M2-pyruvate kinase levels in patients with colorectal cancer. *Turk J Gastroenterol* 24(1):36–42
- Ervens J, Fuchs H, Niemann VT, Hoffmeister B (2008) Pyruvate kinase isoenzyme M2 is not of diagnostic relevance as a marker for oral cancer. *J Craniomaxillofac Surg* 36(2):89–94
- Garon EB, Christofk HR, Hosmer W et al (2014) Dichloroacetate should be considered with platinum-based chemotherapy in hypoxic tumors rather than as a single agent in advanced non-small cell lung cancer. *J Cancer Res Clin Oncol* 140(3):443–452
- Goonetilleke KS, Mason JM, Siriwardana P, King NK, France MW, Siriwardena AK (2007) Diagnostic and prognostic value of plasma tumor M2 pyruvate kinase in periampullary cancer - Evidence for a novel biological marker of adverse prognosis. *Pancreas* 34 (3):318–324
- Hardt PD, Ewald N (2008) Tumor M2 pyruvate kinase: a tumor marker and its clinical application in gastrointestinal malignancy. *Expert Rev Mol Diagn* 8 (5):579–585
- Hardt PD, Toepler M, Ngoumou B, Rupp J, Kloe HU (2003) Measurement of fecal pyruvate kinase type M2 (Tumor M2-PK) concentrations in patients with gastric cancer, colorectal cancer, colorectal adenomas and controls. *Anticancer Res* 23(2A):851–853
- Kobierzycki C, Pula B, Werynska B, Piotrowska A, Muszczynska-Bernhard B, Dziegiel P, Rakus D (2014) The Lack of evidence for correlation of pyruvate kinase M2 expression with tumor grade in non-small cell lung cancer. *Anticancer Res* 34 (7):3811–3817
- Kumar Y, Gurusamy K, Pamecha V, Davidson BR (2007) Tumor M2-pyruvate kinase as tumor marker in exocrine pancreatic cancer; a meta-analysis. *Pancreas* 35 (2):114–119
- Kumar Y, Pinedo IR, Tapuria N, Zabron A, Davidson BR (2008) A comparison of tumour M2-PK with carcinoembryonic antigen and CA19-9 in patients undergoing liver resection for colorectal metastases. *Eur J Gastroenterol Hepatol* 20(10):1006–1011
- Li R, Liu J, Xue H, Huang G (2012) Diagnostic value of fecal tumor M2-pyruvate kinase on colorectal cancer: a meta-analysis. *Int J Cancer* 131(8):1837–1845
- Meng MB, Wang HH, Guo WH, Wu ZQ, Zeng XL, Zaorsky NG, Shi HS, Qian D, Niu ZM, Jiang B, Zhao LJ, Yuan ZY, Wang P (2015) Targeting pyruvate kinase M2 contributes to radiosensitivity of non-small cell lung cancer cells in vitro and in vivo. *Cancer Lett* 356(2):985–993
- Mirsadraee S, Oswal D, Alizadeh Y, Caulo A, van Beek EJR (2012) The 7th lung cancer TNM classification and staging system: Review of the changes and implications. *World J Radiol* 4(4):128–134
- Mountain CF (1997) Revisions in the International System for Staging Lung Cancer. *Chest* 111 (6):1710–1717
- Papadaki C, Sfakianaki M, Lagoudaki E, Giagkas G, Ioannidis G, Trypaki M, Tsakalaki E, Voutsina A, Koutsopoulos A, Mavroudis D, Georgoulis V, Souglakos J (2014) PKM2 as a biomarker for chemosensitivity to front-line platinum-based chemotherapy in patients with metastatic non-small-cell lung cancer. *Br J Cancer* 111(9):1757–1764
- Peng XC, Gong FM, Zhao YW, Zhou LX, Xie YW, Liao HL, Lin HJ, Li ZY, Tang MH, Tong AP (2011) Comparative proteomic approach identifies PKM2 and cofilin-1 as potential diagnostic, prognostic and therapeutic targets for pulmonary adenocarcinoma. *PLoS ONE* 6(11), e27309. doi:10.1371/journal.pone.0027309
- Roudier E, Perrin A (2009) Considering the role of pyruvate in tumor cells during hypoxia. *Biochim Biophys Acta* 1796(2):55–62
- Schneider J, Schulze G (2003) Comparison of tumor M2-pyruvate kinase (tumor M2-PK), carcinoembryonic antigen (CEA), carbohydrate antigens CA 19–9 and CA 72–4 in the diagnosis of gastrointestinal cancer. *Anticancer Res* 23 (6D):5089–5093
- Schulze G (2000) The tumor marker tumor M2-PK: An application in the diagnosis of gastrointestinal cancer. *Anticancer Res* 20(6D):4961–4964
- Voorzanger-Rousselot N, Garnero P (2007) Biochemical markers in oncology. Part I: Molecular basis. Part II: Clinical uses. *Cancer Treat Rev* 33(3):230–283
- Warburg O (1956) Origin of cancer cells. *Science* 123 (3191):309–314
- Zhang B, Chen JY, Chen DD, Wang GB, Shen P (2004) Tumor type M-2 pyruvate kinase expression in gastric cancer, colorectal cancer and controls. *World J Gastroenterol* 10(11):1643–1646

***Clostridium Difficile* Infection Due to Pneumonia Treatment: Mortality Risk Models**

M. Chmielewska, K. Zycinska, B. Lenartowicz,
M. Hadzik-Błaszczyk, M. Cieplak, Z. Kur, and
K.A. Wardyn

Abstract

One of the most common gastrointestinal infection after the antibiotic treatment of community or nosocomial pneumonia is caused by the anaerobic spore *Clostridium difficile* (*C. difficile*). The aim of this study was to retrospectively assess mortality due to *C. difficile* infection (CDI) in patients treated for pneumonia. We identified 94 cases of post-pneumonia CDI out of the 217 patients with CDI. The mortality issue was addressed by creating a mortality risk models using logistic regression and multivariate fractional polynomial analysis. The patients' demographics, clinical features, and laboratory results were taken into consideration. To estimate the influence of the preceding respiratory infection, a pneumonia severity scale was included in the analysis. The analysis showed two statistically significant and clinically relevant mortality models. The model with the highest prognostic strength entailed age, leukocyte count, serum creatinine and urea concentration, hematocrit, coexisting neoplasia or chronic obstructive pulmonary disease. In conclusion, we report on two prognostic models, based on clinically relevant factors, which can be of help in predicting mortality risk in *C. difficile* infection, secondary to the antibiotic treatment of pneumonia. These models could be useful in preventive tailoring of individual therapy.

Keywords

Clostridium difficile • Infection • Mortality risk • Pneumonia • Prognostic model

M. Chmielewska, K. Zycinska (✉), B. Lenartowicz, M. Hadzik-Błaszczyk, M. Cieplak, Z. Kur, and K.A. Wardyn
Department of Family Medicine with Internal and Metabolic Diseases, Medical University of Warsaw, 19/25 Stępińska Street, 00-739 Warsaw, Poland
e-mail: kzycinska@poczta.fm

1 Introduction

One of the most common gastrointestinal infection after antibiotic treatment of community or

hospital acquired pneumonia is caused by *Clostridium difficile* (*C. difficile*) (Becerra et al. 2015; Habayeb et al. 2015). The most important virulence factors of this anaerobic spore-forming bacteria are toxins A and B which are monoglycosyltransferases that damage the gut epithelium (Monot et al. 2015). In addition to broad-spectrum antibiotics, anti-neoplastic agents, proton pump inhibitors, and several other drugs have been reported to induce intestinal dysbiosis, which is part of the pathogenesis of *C. difficile* infection (CDI) (Gabriel and Beriot-Mathiot 2014; Bagdasarian et al 2015). The diagnosis is based on enzyme immunoassays to detect toxins, polymerase chain reaction (PCR) test for toxin genes, single step PCR on a liquid stool sample, or a stool culture of toxin producing spores (Khanna and Pardi 2010). Over the last two decades, there has been an increased incidence and severity of CDI, due mainly to the emergence of new strain variants (Monot et al. 2015; Jawa and Mercer 2012). Clinical spectrum of the disease is wide, ranging from an asymptomatic carriers, through mild self-limiting diarrhea, to pseudo-membranous colitis (Sun and Hirota 2015). Although the risk of CDI increases with duration of treatment, the disease can also occur after a short treatment course. It is essential to assess the severity of CDI at its onset to combat it more effectively and prevent progression into the fulminant form. In complicated CDI cases, colonic inflammation and tissue disruption can lead to toxic megacolon, a condition requiring urgent surgical intervention (Dubberke et al. 2008). The overall mortality rate in CDI ranges from 5.5 to 6.9 %, but it can reach about 17 % in severe outbreaks (Zilberberg et al. 2008). In the US, *C. difficile* is one of the leading gastrointestinal causes of death in nosocomial infections (Peery et al 2012). Studies show that CDI severity depends on the preceding infectious condition treated with antibiotics. The most common are urinary tract infections and lower respiratory tract infections. Further, there are reports demonstrating that CDI following in-hospital pneumonia treatment is connected with a higher mortality than in other causes of hospitalization (Becerra et al. 2015; Polgreen et al. 2007; Pépin

et al. 2005). Therefore, the aim of this study was to assess the strength of mortality risk models of *C. difficile* infection in patients with antecedent treatment for pneumonia in order to improve the ability to counteract the most severe course of the disease in a timely manner.

2 Methods

The study was approved by a local Ethics Committee of the Medical University of Warsaw, Poland. We conducted a retrospective analysis of 217 nosocomial or community-acquired pneumonia patients with subsequent *C. difficile* infection, hospitalized at the Internal Medicine Ward of the Czerniakowski Hospital in Warsaw between May 2012 and February 2015. The diagnosis of pneumonia was based on clinical signs and symptoms such as productive cough and fever, along with radiological findings indicating pneumonia. The CDI was defined as diarrhea with the positive result of a stool assay for the glutamate dehydrogenase antigen, along with the presence of toxins A and B confirmed by an enzyme immunoassay or stool culture. Diarrhea was defined as a passage of three or more unformed stools in a 24-h period (Kelly and LaMont 2008). The inclusion criterium was antibiotic treatment for pneumonia within a two-month period preceding the CDI diagnosis. Clinical features taken into account consisted of temperature, blood biochemistry (leukocyte, neutrophil, and red blood cell counts, hemoglobin, hematocrit, C-reactive protein, and serum creatinine, urea, and albumin levels), abdominal tenderness or ileus, hypotension, duration of symptoms, and mental status. Additional features assessed included comorbidities, patient residence, and the kind of antibiotic treatment. The score of Pneumonia Severity Index (PSI) was assessed for community-acquired pneumonia, APACHE II for nosocomial pneumonia, and CURB-65 for all patients. The assessment was performed at onset of CDI.

Continuous data were reported as means \pm SD and categorical data as percent. A univariate analysis was employed to assess the relevance of

continuous variables for mortality risk. Odds ratios (OR) with 95 % confidence intervals (95 %CI) were calculated for the logistic regression analysis. To create a mortality risk model in patients treated for pneumonia, logistic regression analysis and multivariate fractional polynomials were employed. Statistical significance of risk models was assessed with a Chi-squared test and an analysis of residuals. The quality of models was expressed by three conditions: low classification error for a learning sample, area under the receiver operating curve (ROC), and continuity of the same classification error in a cross-validation method (Ratner 2011; Royston and Altman 2010; Hosmer et al. 2004; Sauerbrei and Royston 1999). An alpha level of <0.05 was considered statistically significant. The analyses were performed using R Statistical Software, ver. 3.1.2 (GNU General Public License).

3 Results

Ninety four (43.3 %) out of the 217 patients with *C. difficile* infection had post-pneumonia infection. Fifty (53.2 %) of these patients went through severe or severe and complicated CDI, which stands for 51 % of all severe cases of CDI irrespective of the background disease. For 21 (22.3 %) post-pneumonia patients *C. difficile* infection was fatal. In the univariate analysis, the mortality risk factors were the following: age (OR 1.084; 95 %CI 1.038–1.138), duration of symptoms (OR 1.156; 95 %CI 1.067–1.261), leukocyte count (OR 1.062; 95 %CI

1.023–1.107), neutrophil count (OR 1.065; 95 %CI 1.025–1.113), C-reactive protein (OR 1.076; 95 %CI 1.025–1.131), serum urea (OR 1.015; 95 %CI 1.008–1.022), peak serum creatinine level (OR 1.498; 95 %CI 1.124–2.029). To create a mortality risk model, we also took into account categorical variables characterizing patients. The multivariate fractional polynomials revealed two statistically significant and clinically relevant mortality risk models. The first model includes the following features: patient’s age, white blood cells count, serum creatinine concentration, hematocrit, and coexisting neoplasia and COPD. The second model includes CURB-65 score, intake of more than one antibiotics, serum urea and creatinine levels, and altered mental status. These two mortality risk models met the criteria of statistical significance and quality (Table 1). The other factors selected for mortality modeling, namely fever, C-reactive protein, serum albumin, intake of proton pump inhibitors, hospitalization in intensive care unit, and PSI and APACHE II scores turned out to be of insignificant meaning in any configuration.

4 Discussion

The present study emphasizes an essential role of CDI risk factors in mortality prediction. We primarily focused on the group of patients who were treated for pneumonia, since the disease contributed to nearly half of CDI and more than half of severe cases. The mortality models show that the pulmonary condition such as COPD or

Table 1 Mortality risk models

Mortality risk model I		Mortality risk model II	
Variable	p-value	Variable	p-value
Age	0.01	CURB-65	0.03
White blood cells	0.01	Intake of more than 1 antibiotics	0.05
Creatinine serum level	0.002	Creatinine serum level	0.01
Hematocrit	0.01	Urea serum level	0.01
Coexisting neoplasia	0.04	Altered mental status	0.03
Coexisting COPD	0.01		
AUC – 0.933		AUC – 0.969	

COPD chronic obstructive pulmonary disease, AUC area under curve in a receiver operating characteristic

the result of CURB-65 scoring play a significant role in the estimation of mortality. The study revealed the lack of influence of PSI and APACHE II scoring on shaping the mortality models.

C. difficile infection is a potential life-threatening complication after the antibiotic therapy. There is an increase in the incidence and mortality attributed to CDI globally. Over the past decade, the number of *C. difficile*-associated megacolon cases has nearly tripled, and the mortality rate associated with this condition has doubled (Kuy et al. 2016). Moreover, the epidemiology of CDI has appreciably changed, with the increasing occurrence of community-acquired CDI (Goudarzi et al. 2014). It is estimated that pneumonia treatment stands for the risk factor of severe and complicated CDI. Therefore, it is essential to evaluate the presence of risk factors in pneumonia patients in order to predict mortality and to choose proper diagnostic methods and treatment (Becerra et al. 2015). Although metronidazole is the first-line choice for CDI treatment, the drug has limited efficacy in severe cases. In such cases vancomycin provides better, both initial and sustained, cure rates but its use increases resistance to its therapeutic effectiveness among *Enterococcus* spp. Therefore, vancomycin treatment ought to be carefully weighed up and applied in severe CDI only (Di et al. 2015). In patients with a risk of recurrence and the necessity for a long-term treatment, fidaxomicin or fecal microbiota transplant should be considered to prevent reinfections (Nathwani et al. 2014; Silverman et al. 2010).

CDI therapy should be carefully selected taking into account its severity and mortality risks. Otherwise, patients are put on increased risk of complications, recurrence, mortality, and the disease generates extra healthcare costs (Johnson 2009; Kelly et al. 1994). The estimation of mortality risks enables to better predict fatal complications and prevent them from happening using proper prophylaxis, such as dietary choices or albumin supplementation (Surawicz et al. 2013). In conclusion, the study demonstrates two mortality models for *C. difficile* infection,

which employ clinically relevant factors, in patients treated for pneumonia. These models can presage the way the CDI progresses, which helps undertake the most appropriate treatment decisions.

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

References

- Bagdasarian N, Krishna R, Preeti NM (2015) Diagnosis and treatment of *Clostridium difficile* in adults: a systematic review. *JAMA* 313(4):398–408
- Becerra MB, Becerra BJ, Banta JE, Safdar N (2015) Impact of *Clostridium difficile* infection among pneumonia and urinary tract infection hospitalization: an analysis of the Nationwide Inpatient Sample. *BMC Infect Dis* 15:254
- Di X, Bai N, Zhang X, Liu B, Ni W, Wang J, Wang K, Liang B, Liu Y, Wang R (2015) A meta-analysis of metronidazole and vancomycin for the treatment of *Clostridium difficile* infection, stratified by disease severity. *Braz J Infect Dis* 19(4):339–349
- Dubberke ER, Butler AM, Reske KA, Agniel D, Olsen MA, D'Angelo G, McDonald LC, Fraser VJ (2008) Attributable outcomes of endemic *Clostridium difficile*-associated disease in nonsurgical patients. *Emerg Infect Dis* 14(7):1031–1038
- Gabriel L, Beriot-Mathiot A (2014) Hospitalization stay and costs attributable to *Clostridium difficile* infection: a critical review. *J Hosp Infect* 88(1):12–21
- Goudarzi M, Seyedjavadi SS, Goudarzi H, Mehdizadeh Aghdam E, Nazeri S (2014) *Clostridium difficile* infection: epidemiology, pathogenesis, risk factors, and therapeutic options. *Scientifica* (Cairo) 2014:916826. doi:10.1155/2014/916826
- Habayeb H, Sajin B, Patel K, Grundy C, Al-Dujaili A, Van de Velde S (2015) Amoxicillin plus temocillin as an alternative empiric therapy for the treatment of severe hospital-acquired pneumonia: results from a retrospective audit. *Eur J Clin Microbiol Infect Dis* 34(8):1693–1699
- Hosmer DW Jr, Lemeshow S, Sturdivant RX (2004) *Applied logistic regression*, 3rd edn. Wiley, Hoboken. ISBN 978-0-470-58247-3
- Jawa RS, Mercer DW (2012) *Clostridium difficile*-associated infection: a disease of varying severity. *Am J Surg* 204(6):836–842
- Johnson S (2009) Recurrent *Clostridium difficile* infection: a review of risk factors, treatments, and outcomes. *J Infect* 58(6):403–410
- Kelly CP, LaMont JT (2008) *Clostridium difficile* – more difficult than ever. *N Engl J Med* 359(18):1932–1940
- Kelly CP, Pothoulakis C, LaMont JT (1994) *Clostridium difficile* colitis. *N Engl J Med* 330(4):257–262

- Khanna S, Pardi DS (2010) The growing incidence and severity of *Clostridium difficile* infection in inpatient and outpatient settings. *Expert Rev Gastroenterol Hepatol* 4(4):409–416
- Kuy S, Jenkins P, Romero RA, Samra N, Kuy S (2016) Increasing incidence of and increased mortality associated with *Clostridium difficile*-associated megacolon. *JAMA Surg* 151(1):85–86
- Monot M, Eckert C, Lemire A, Hamiot A, Dubois T, Tessier C, Dumoulaud B, Hamel B, Petit A, Lalande V, Ma L, Bouchier C, Barbut F, Dupuy B (2015) *Clostridium difficile*: new insights into the evolution of the pathogenicity locus. *Sci Rep* 5:15023. doi:10.1038/srep15023
- Nathwani D, Cornely OA, Van Engen AK, Odufowora-Sita O, Retsa P, Odeyemi IA (2014) Cost-effectiveness analysis of fidaxomicin versus vancomycin in *Clostridium difficile* infection. *J Antimicrob Chemother* 69(11):2901–2912
- Peery AF, Dellon ES, Lund J, Crockett SD, McGowan CE, Bulsiewicz WJ, Shaheen NJ (2012) Burden of gastrointestinal disease in the United States: 2012 update. *Gastroenterology* 143(5):1179–1187
- Pépin J, Saheb N, Coulombe MA, Alary ME, Corriveau MP, Authier S, Leblanc M, Rivard G, Bettez M, Primeau V, Nguyen M, Jacob CE, Lanthier L (2005) Emergence of fluoroquinolones as the predominant risk factor for *Clostridium difficile*-associated diarrhea: a cohort study during an epidemic in Quebec. *Clin Infect Dis* 41(9):1254–1260
- Polgreen PM, Chen YY, Cavanaugh JE, Ward M, Coffman S, Hornick DB, Diekema DJ, Herwaldt LA (2007) An outbreak of severe *Clostridium difficile*-associated disease possibly related to inappropriate antimicrobial therapy for community-acquired pneumonia. *Infect Control Hosp Epidemiol* 28(2):212–214
- Ratner B (2011) Statistical and machine-learning data mining: techniques for better predictive modeling and analysis of big data, 2nd edn. CRC Press Book, Boca Raton. ISBN 9781439860915
- Royston P, Altman DG (2010) Visualizing and assessing discrimination in the logistic regression model. *Stat Med* 29(24):2508–2520
- Sauerbrei W, Royston P (1999) Building multivariable prognostic and diagnostic models: transformation of the predictors by using fractional polynomials. *J R Stat Soc Ser A Stat Soc* 162:71–94
- Silverman MS, Davis I, Pillai DR (2010) Success of self-administered home fecal transplantation for chronic *Clostridium difficile* infection. *Clin Gastroenterol Hepatol* 8(5):471–473
- Sun X, Hirota SA (2015) The roles of host and pathogen factors and the innate immune response in the pathogenesis of *Clostridium difficile* infection. *Mol Immunol* 63(2):193–202
- Surawicz CM, Brandt LJ, Binion DG, Ananthakrishnan AN, Curry SR, Gilligan PH, McFarland LV, Mellow M, Zuckerbraun BS (2013) Guidelines for diagnosis, treatment, and prevention of *Clostridium difficile* infections. *Am J Gastroenterol* 108(4):478–498
- Zilberberg MD, Shorr AF, Kollef MH (2008) Increase in adult *Clostridium difficile*-related hospitalizations and case-fatality rate, United States, 2000–2005. *Emerg Infect Dis* 14(6):929

Predictors of Progression in IgA Nephropathy in Childhood

M. Mizerska-Wasiak, J. Małydk, A. Turczyn, K. Cichoń-Kawa, A. Rybi-Szumińska, A. Wasilewska, B. Bieniaś, M. Zajączkowska, M. Miklaszewska, J. Pietrzyk, U. Demkow, M. Roszkowska-Blaim, and M. Pańczyk-Tomaszewska

Abstract

The aim of this retrospective study was to assess the usefulness of potential predictors of poor prognosis in IgA nephropathy in children. The study population consisted of 55 children aged 11 ± 4 years, diagnosed on the basis of the Oxford classification and MEST score of kidney biopsy findings. Proteinuria, glomerular filtration rate (GFR), and the IgA/C3 serum ratio were assessed in all patients twice: at onset and at follow-up. The patients were treated with steroids, immunosuppressive drugs, and/or angiotensin-converting enzyme inhibitors. Follow-up was at 3.9 ± 2.9 (median 2.7) years. The patients were subdivided into two groups: with GFR <90 and ≥ 90 mL/min at follow-up. ROC AUC curves and logistic regression were used to evaluate the power of prognostic factors. The two groups did not differ regarding the level of proteinuria, MEST score, and the IgA/C3 ratio at onset of disease. There was a significant association between GFR reductions at onset and follow-up (AUC = 0.660; $p < 0.05$). In patients with nephrotic range proteinuria at

M. Roszkowska-Blaim (deceased)

M. Mizerska-Wasiak (✉), A. Turczyn,
M. Roszkowska-Blaim, and M. Pańczyk-Tomaszewska
Department of Pediatrics and Nephrology, Medical
University of Warsaw, 63A Zwirki i Wigury St., Warsaw
02-091, Poland
e-mail: wasiaczki@wp.pl

J. Małydk
Department of Pathology, Medical University of Warsaw,
Warsaw, Poland

K. Cichoń-Kawa
Student Research Group at the Department of Pediatrics
and Nephrology, Medical University of Warsaw,
Warsaw, Poland

A. Rybi-Szumińska and A. Wasilewska
Department of Pediatrics and Nephrology, Medical
University of Białystok, Białystok, Poland

B. Bieniaś and M. Zajączkowska
Department of Pediatric Nephrology, Medical University
of Lublin, Lublin, Poland

M. Miklaszewska and J. Pietrzyk
Department of Pediatric Nephrology, Jagiellonian
University of Cracow, Cracow, Poland

U. Demkow
Department of Laboratory Diagnostics and Clinical
Immunology of Developmental Age, Medical University
of Warsaw, Warsaw, Poland

onset, proteinuria at follow-up was more frequent compared with other patients (AUC = 0.760; $p < 0.05$), MEST score ≥ 3 tended to be associated with reduced GFR (AUC = 0.650; $p = 0.07$) but not with proteinuria (AUC = 0.608; $p = 0.47$), and the IgA/C3 ratio was higher ($p < 0.05$) at follow-up. No significant associations were found between the IgA/C3 ratio at onset and reduced GFR (AUC = 0.565; $p = 0.46$) or proteinuria at follow-up (AUC = 0.263; $p = 0.20$). We conclude that predictors of poor outcome in childhood IgAN include the following: GFR reduction, nephrotic range proteinuria at onset of disease, and high MEST score in Oxford classification of kidney biopsy. Despite a higher serum IgA/C3 ratio in children with impaired renal function in long-term follow-up, we failed to demonstrate a significant association between this ratio at onset of disease and reduced GFR or persistent proteinuria at follow-up. Thus, IgA/C3 ratio is not a good foreteller of progression of IgA nephropathy in childhood.

1 Introduction

IgA nephropathy (IgAN) is the most common type of glomerulonephritis worldwide. The diagnosis is based on renal biopsy which shows predominant characteristic IgA deposits, often accompanied by complement component C3 deposits (Coppo and D'Amico 2005; Schena 1990; Berger and Hinglais 1968). The key element of the pathogenesis of IgAN is impaired glycosylation of IgA, resulting in reduced hepatic clearance of galactose-deficient (GD) IgA1, its deposition within the mesangium, and initiation of an autoimmune inflammatory response (Suzuki et al. 2011). In children with IgAN, serum IgA level is normal or elevated. Elevated IgA level has been reported in 16 % of cases (Yoshikawa et al. 2001), and in 50 % of cases in our own material (Mizerska-Wasiak et al. 2015).

Serum C3 levels are usually normal or slightly increased despite significant elevation of C3 breakdown products in the plasma and the evidence of C3 activation in the glomerular mesangium (Komatsu et al. 2004; Wyatt et al. 1987). Complement activation occurs usually *via* alternative pathways. One such pathway is the lectin pathway activated by the binding of

mannose-binding lectin (MBL) and ficolins to carbohydrate ligands, which is associated with a more severe disease course (Roos et al. 2006). An important prognostic factor is the result of the Oxford classification of IgAN based on kidney biopsy, in which the presence of mesangial proliferation (M), endocapillary hypercellularity (E), sclerosis (S), and tubular atrophy/interstitial fibrosis (T) is considered to portend a poor prognosis (Working Group of the International IgA Nephropathy Network and the Renal Pathology Society et al. 2009).

The serum IgA/C3 ratio also is used as a marker of IgAN prognosis. High values of this ratio enable to differentiate between IgAN and other glomerulopathies (Tomino 2003; Tomino et al. 2000). Komatsu et al. (2004) have shown that the cut-off IgA/C3 serum ratio greater than 4.5 is associated with worse outcomes in adults. In children, serum IgA/C3 ratio may be helpful in predicting the MEST score (a sum of M + E + S + T in the Oxford classification) in kidney biopsy, with IgA/C3 greater than 2.26 being associated with the MEST score >3 (Mizerska-Wasiak et al. 2015). The aim of the present study was to assess the risk of progression of IgAN as based on the serum IgA/C3 ratio in children at onset of disease.

2 Methods

The study was approved by a local Ethics Committee of the Medical University in Warsaw, Poland. This is a retrospective study that included 55 children aged 11 ± 4 years, with complete disease files, out of the 118 children with IgAN reported in the Polish Pediatric IgAN Registry. The IgAN was diagnosed on the basis of renal biopsy. The following data were analyzed: age at onset of IgAN, proteinuria, hematuria, glomerular filtration rate (GFR), and the serum IgA/C3 ratio assessed twice, at onset of disease and at follow-up. Proteinuria was measured in a 24-h urine collection using the Exton turbidimetric method and was expressed in mg/kg/day. The nephrotic range proteinuria was defined as ≥ 50 mg/kg/day, and proteinuria < 50 mg/kg/day was considered a non-nephrotic range proteinuria. Hematuria was established as > 5 erythrocytes *per* field of view under a microscope, and gross hematuria was defined as the macroscopic presence of blood in urine. The Schwartz formula was used to calculate GFR (Schwartz et al. 2009). Serum IgA and C3 levels were measured using the nephelometric method.

The histological features were scored according to the Oxford classification (M-mesangial hypercellularity, E-endocapillary hypercellularity, S-segmental sclerosis, T-tubular atrophy/interstitial fibrosis; absent = 0, present = 1). The MEST score was the sum of M + E + S + T (0–4). In addition, the presence of crescents was analyzed in the biopsy samples. Renal biopsies were evaluated by local pathologists and later verified at the Department of Pathology of the Medical University of Warsaw in Poland.

Following the diagnosis, the patients were treated with steroids alone or in combination with the immunosuppressive drugs: azathioprine (AZA), cyclophosphamide (CFX), and cyclosporine A (CsA), and with the angiotensin-converting enzyme inhibitors (ACEI) or angiotensin receptor blockers (ARB). In the

patients who required more than one treatment regimen, we analyzed the first-choice treatment and the second-choice treatment which was used when the former was deemed unsuccessful. The treatment was considered unsuccessful if no reduction in proteinuria and no improvement in GFR were seen.

We divided the patients into two groups depending on GFR at follow-up, with renal dysfunction taken as the distinguishing point: Group A (GFR < 90 mL/min) and Group B (GFR ≥ 90 mL/min). In addition, we analyzed the subgroups with and without proteinuria at the end of follow-up. Based on the number of adverse prognostic factors in the Oxford classification, the patients were also analyzed in the subgroups with the MEST score ≥ 3 and < 3 .

2.1 Statistical Evaluation

Results were expressed as mean values with the standard deviation, and median and ranges. The receiver-operating characteristic (ROC) curves were used to categorize patient according to the study paradigm, i.e., normal or abnormal GFR, serum IgA/C3 ratio, and the patient subgroups. The area under the curve (AUC) > 0.5 with $p < 0.05$ were considered statistically significant. Sensitivity, specificity, and the optimal cut-off values were determined. The variance of the AUC was determined using the Bamber method. ROC curves were analyzed using the XLSTAT statistical package.

Logistic regression was used to evaluate the strength of the relationship between a dichotomous variable and quantitative and qualitative variables. Significance of the relationship was evaluated using the Wald chi-squared test. The odds ratio and confidence intervals were calculated for significant parameters. Logistic regression analysis was performed using a commercial Statistica ten package (StatSoft, Tulsa, OK). A multivariate analysis was performed to determine the adverse prognostic factors.

3 Results

The patients' characteristics are shown in Table 1. Among the 55 patients investigated, nephrotic-range proteinuria was found at disease onset in 11 patients (20 %), and non-nephrotic range proteinuria in 29 patients (53 %). Hematuria was present in all patients. A reduced GFR

(<90 mL/min) was found at disease onset in 22 (40 %) children, including two with nephrotic-range proteinuria, 13 with non-nephrotic range proteinuria, and seven with isolated hematuria. The GFR was normal in 33 (60 %) patients. The mean serum IgA/C3 ratio in the entire study group was 2.44 ± 1.23 , and values above the cut-off level of 2.26 were noted in 28 (51 %) children.

Renal biopsy was performed, on average, 0.9 ± 1.3 (median 0.4) years after onset of disease. The findings according to the Oxford classification were the following: M1 in 87 % of patients, E1 in 20 % of patients, S1 in 31 % of patients, and T1 in 16 % of patients, with none of the patients showing the T2 stage. The crescents were seen on renal biopsy in 18 % of patients. In the entire study group, the MEST score of one was noted significantly more frequently ($p < 0.05$) than the other MEST score values.

The most common first-choice treatment was a combination of corticosteroids and azathioprine with and without ACEI/ARB, used in 37 % of the patients when proteinuria and the MEST score ≥ 1 were present. Nephroprotective therapy only (ACEI/ARB) was used in 28 % of the patients, and steroid therapy with and without ACEI/ARB was used in 19 % of patients. Other immunosuppressive medications, i.e., cyclophosphamide and cyclosporin A, were used in a few cases.

Duration of follow-up in the entire study group was 3.9 ± 2.9 (median 2.7) years. The results of the analysis depending on children's GFR at follow-up, Group A – reduced GFR <90 mL/min and Group B – normal GFR ≥ 90 mL/min are shown in Table 2. The GFR <90 mL/min was found in 16 (29 %) children, including GFR <60 mL/min in one patient. No significant differences were found between Groups A and B regarding the mean age at onset, the duration of follow-up, the mean proteinuria, and the mean serum IgA/C3 ratio. The MEST score in renal biopsy tended to be greater in Group A compared with Group B ($p > 0.05$). The nephroprotective treatment with cyclophosphamide and cyclosporin A were used at a similar rate in both groups. In Group B, a combination of a steroid and azathioprine was used

Table 1 Characteristics of study group at onset of disease ($n = 55$)

Biochemical data	
GFR (mL/min)	94.3 ± 33.8
< 90 mL/min (no of patients; %)	22 (40 %)
Proteinuria (mg/kg/day)	42.1 ± 132.1
≥ 50 mg/kg/day (no of patients; %)	11 (20 %)
IgA/C3 ratio	2.44 ± 1.23
Renal biopsy – Oxford Classification (no of patients; %)	
M1/M0	48 (87 %)/7 (13 %)
E1/E0	11 (20 %)/44 (80 %)
S1/S0	17 (31 %)/38 (69 %)
T1/T0	9 (16 %)/46 (84 %)
c1/c0	10 (18 %)/45 (82 %)
MEST score (no of treatment regimens %)**	
0	4 (7 %)
1	30 (55 %)*
2	13 (24 %)
3	8 (14 %)
Treatment (no of patients; %)	
ACEI/ARB	19 (28 %)
Steroids with & without ACEI/ARB	13 (19 %)
AZA + steroids with & without ACEI/ARB	25 (37 %)
CFX + steroids with & without ACEI	7 (11 %)
CsA+ GCS	3 (5 %)

GFR glomerular filtration rate, M mesangial proliferation, E endocapillary hypercellularity, S sclerosis, T tubular atrophy/interstitial fibrosis, c crescents, AZA azathioprine, CFX cyclophosphamide, CsA cyclosporine A, ACEI angiotensin-converting enzyme inhibitor, ARB angiotensin receptor blocker

* $p < 0.05$ vs. other MEST scores

**one patient can be treated by more than one treatment regimen

Table 2 Study findings in children with IgAN in relation to GFR at follow-up

	Group A (<i>n</i> = 16) GFR <90 mL/min	Group B (<i>n</i> = 39) GFR ≥90 mL/min	<i>p</i>
Age at onset (year)	12.6 ± 2.6	10.4 ± 4.4	ns
Duration of follow-up (year)	4.1 ± 2.72	3.8 ± 3.0	ns
GFR at onset (mL/min)	92.1 ± 37.6	95.4 ± 32.6	ns
GFR at follow-up (mL/min)	76.6 ± 11.0	112.0 ± 18.6	<0.0001
Proteinuria at onset (mg/kg/day)	20.2 ± 32.4	51.1 ± 155.2	ns
Proteinuria at follow-up (mg/kg/day)	6.2 ± 14.1	4.7 ± 12.1	ns
No proteinuria (no of patients; %)	11 (69 %)	28 (72 %)	ns
IgA/C3 ratio at onset	2.69 ± 1.40	2.33 ± 1.19	ns
IgA/C3 ratio at follow-up	2.71 ± 1.14	2.34 ± 1.11	ns
MEST score	1.8 ± 1.1	1.4 ± 0.9	ns
c1 (no of patients; %)	2 (13 %)	8 (21 %)	ns
Treatment (no of treatment regimens %)			
ACEI/ARB	8 (38 %)	11 (24 %)	ns
Steroids and/or ACEI/ARB	8 (38 %)	5 (11 %)*	<0.05
AZA + steroids and/or ACEI/ARB	3 (14 %)	22 (48 %)*	<0.05
CFX + steroids and/or ACEI	1 (5 %)	6 (13 %)	ns
CsA+ steroids	1 (5 %)	2 (4 %)	ns

GFR glomerular filtration rate, *c* crescents, AZA azathioprine, CFX cyclophosphamide, CsA cyclosporine A, ACEI angiotensin-converting enzyme inhibitor, ARB angiotensin receptor blocker

significantly more frequently ($p < 0.05$) compared with Group A, while in the latter, steroids with and without ACEI were used more frequently compared with group B ($p < 0.05$). No significant differences were found between the two groups in the mean proteinuria and serum IgA/C3 ratio at follow-up, although the ratio tended to be greater in Group A.

In the patients of combined groups with reduced and unreduced GFR, ROC curve analysis was performed to assess whether the serum IgA/C3 ratios at onset and at follow-up were associated with reduced GFR at follow-up. As demonstrated by the AUC values, no such associations were found; the IgA/C3 ratio at onset and reduced GFR at follow-up (AUC = 0.565; $p = 0.46$) (Fig. 1) and the IgA/C3 ratio at follow-up and reduced GFR at follow-up (AUC = 0.607; $p = 0.19$) (Fig. 2). However, we found a significant relationship between reduced GFR at onset and reduced GFR at follow-up (AUC = 0.660, $p < 0.05$).

The MEST score ≥ 3 in renal biopsy tended to be associated with a reduced GFR at follow-up (AUC = 0.650; $p = 0.07$). The patients with nephrotic-range proteinuria at onset had

proteinuria at follow-up significantly more frequently than the other patients had (AUC = 0.760; $p < 0.05$). However, MEST score ≥ 3 was unassociated with proteinuria at follow-up (AUC = 0.608; $p = 0.47$). The serum IgA/C3 ratio was greater at follow-up in children with nephrotic proteinuria at onset, but this trend was insignificant (AUC = 0.247; $p < 0.05$). No significant associations were found between the IgA/C3 ratio at onset and GFR at follow-up (AUC = 0.565; $p = 0.47$) or nephrotic proteinuria at follow-up (AUC = 0.263; $p = 0.02$). In the patients with M1 and S1 in renal biopsy, serum IgA/C3 ratios at follow-ups were >1.97 , which was of a significant diagnostic value (AUC = 0.742 and AUC = 0.686, respectively, $p < 0.05$).

The findings regarding IgAN in relation to MEST score are presented in Table 3. The patients with MEST score ≥ 3 presented a significantly higher proteinuria at onset and more frequently had crescents in renal biopsy compared with those having MEST score < 3 ($p < 0.05$). No significant relationships were found between MEST scores 0–2 and 3–4 and GFR at follow-up, although the AUC was 0.650 ($p = 0.07$). Nor

Fig. 1 ROC curve for the relation between IgA/C3 ratio at onset of disease and reduced glomerular filtration rate (GFR) at follow-up

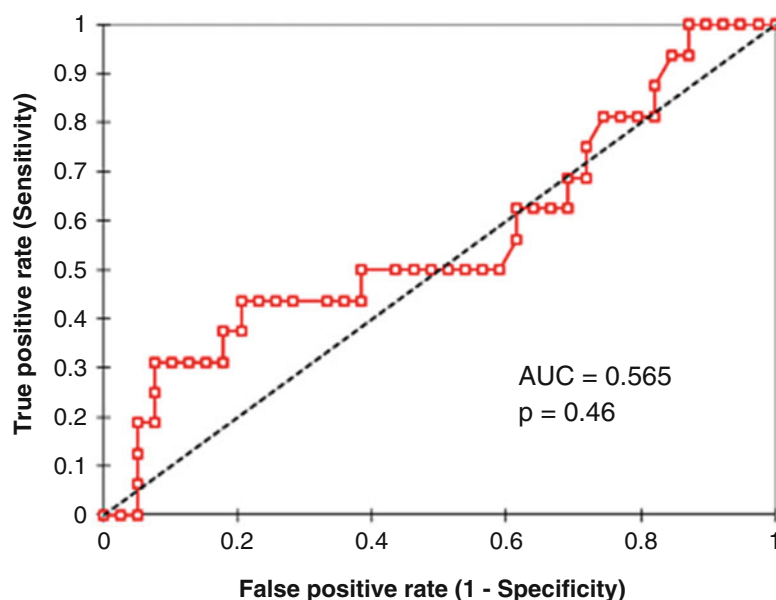
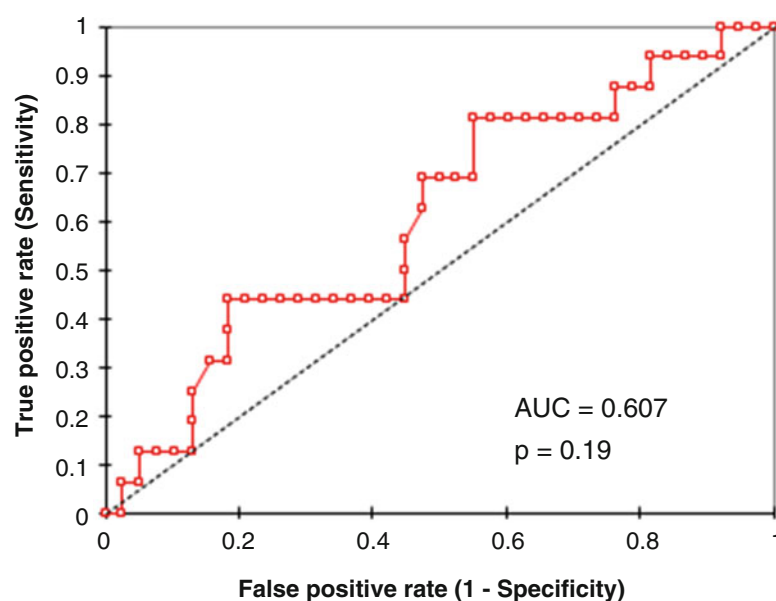


Fig. 2 ROC curve for the relation between IgA/C3 ratio at follow-up and reduced glomerular filtration rate (GFR) at follow-up



was there any appreciable association between MEST score and IgA/C3 ratio at follow-up (AUC = 0.630; $p = 0.12$).

Finally, taking proteinuria as the endpoint, we compared patients with proteinuria >150 mg/day at follow-up and those without proteinuria at the end of follow-up. There were no significant differences between both groups in terms of the

mean GFR at the beginning and end of follow-up. Likewise, mean proteinuria did not differ between these groups at the beginning of follow-up, nor was there any difference in the mean IgA/C3 ratio at disease onset and at follow-up between these two groups. The MEST score in renal biopsy was significantly higher in the patient group with proteinuria compared with

Table 3 Study findings in children with IgAN in relation to MEST score

	MEST score ≥ 3 ($n = 8$)	MEST score < 3 ($n = 47$)	p
Age at onset (year)	13.5 \pm 2.9	11.2 \pm 3.9	ns
Age at follow-up (year)	16.6 \pm 2.1	15.8 \pm 4.0	ns
GFR at onset (mL/min)	74.6 \pm 34.1	97.8 \pm 32.7	ns
GFR at follow-up (mL/min)	88.9 \pm 22.5	103.9 \pm 22.9	ns
Proteinuria at onset (mg/kg/day)	158.9 \pm 328.1	22.2 \pm 36.3	p < 0.01
Proteinuria at follow-up (mg/kg/day)	12.7 \pm 18.3	3.9 \pm 11.1	p = 0.06
IgA/C3 ratio at onset	2.98 \pm 1.80	2.30 \pm 1.10	ns
IgA/C3 ratio at follow-up	2.89 \pm 1.54	2.37 \pm 1.04	ns
c1 (no of patients, %)	5 (62 %)	5 (11 %)	p < 0.05
ACEI/ARB	2 (25 %)	17 (36 %)	ns
Steroids and/or ACEI/ARB	3 (37 %)	10 (1 %)	ns
AZA + steroids and/or ACEI/ARB	4 (50 %)	22 (46 %)	ns
CFX + steroids and/or ACEI/ARB	3 (37 %)	2 (4 %)	p < 0.05
CsA + steroids	1 (12 %)	2 (4 %)	ns

GFR, glomerular filtration rate; c, crescents; AZA, azathioprine; CFX, cyclophosphamide; CsA, cyclosporine A; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker

that without proteinuria (2.0 ± 1.2 vs. 1.3 ± 0.77 ; $p < 0.01$). Multivariate analysis showed no association between the IgA/C3 ratio and GFR reduction below 90 mL/min at follow-up.

4 Discussion

In the present study, clinical symptoms at onset of IgAN were similar to those observed in a Japanese study (Yoshikawa et al. 2001), although we observed GFR reduction below 90 mL/min in 40 % of children and below 60 mL/min in 8 children (14 %), while in the Japanese study nephrotic or nephritic syndrome with reduced GFR was present in merely 12 % of children. The mean serum IgA/C3 ratio in our pediatric IgAN population was lower than that suggested in adult studies as allowing differentiation between IgAN and other nephropathies; for instance >3.01 reported by Maeda et al. (2003). Concerning the Oxford classification of renal biopsy specimens, we found that M1 was the most common (70 %) and S1 was less frequent. In contrast, S1 was the most common finding (70 %) and M1 was seen in just 28 % of patients in the VALIGA study (Coppo et al. 2014). We also found that the MEST score of 1 was significantly more frequent compared with the other

MEST score values, which may be associated with a relatively early accomplishment of renal biopsy, with the median time from disease onset to biopsy of 0.4 years. Our previous studies indicate that the MEST score may be estimated from the IgA/C3 ratio at onset of disease (Mizerska-Wasiak et al. 2015).

Due to the lack of standards regarding the management of children with IgAN, therapy used in various clinical centers depends mostly on the severity of proteinuria and the histopathological findings in renal biopsy. The most common drug regimen includes azathioprine combined with corticosteroids and ACEI/ARB. In the present study, a combination of azathioprine and corticosteroids was used significantly more frequently in children who exhibited normal GFR at follow-up, which is suggestive of a good amelioration of renal dysfunction with this treatment compared with a regimen of corticosteroids with or without ACEI/ARB, which was used more frequently in children with reduced GFR at follow-up. Treatment with azathioprine has also been successfully employed in a Japanese pediatric population (Yoshikawa et al. 2001).

In the VALIGA study, immunosuppression has been associated with a significant reduction in proteinuria, a slower rate of renal function decline, and a greater renal survival. Using the propensity score, the authors matched

184 subjects who received corticosteroids and renin-angiotensin system blockers with another group of 184 patients with a similar risk profile of progression who received only renin-angiotensin system blockers. In the corticosteroid group, proteinuria and renal function decline were reduced, renal survival was increased. The benefits extended to patients with an estimated GFR of ≤ 50 mL/min per 1.73 m^2 , and these benefits increased in proportion to the improvement in proteinuria. Thus, corticosteroids reduce risk of disease progression regardless of initial GFR (Tesar et al. 2015).

In the present study, ROC curve analysis confirmed the presence of an association between GFR reduction at follow up and GFR reduction at onset of disease, which is an established adverse prognostic factor. This association was confirmed despite a shorter period of follow-up in our study compared with that in the VALIGA study; medians of 2.7 and 4.7 years, respectively (Tesar et al. 2015). In line with other studies we also demonstrate that nephrotic-range proteinuria is another adverse prognostic factor (Suzuki et al. 2011; Schena 1990; Berger and Hinglais 1968). On the other hand, we observed only a trend for the MEST score ≥ 3 as being a poor prognostic factor, which might possibly be associated with the improvement of histologic lesions by treatment, as is also concluded in a study of Coppo et al. (2014).

The serum IgA/C3 ratio at onset of disease, despite its association with MEST in renal biopsy findings (Mizerska-Wasiak et al. 2015), was not found in the present study to be a significant marker of poor outcomes, i.e., of GFR reduction. The prognostic value of IgA/C3 ratio could also be lessened by treatment used or by insufficient duration of follow-up. We submit, however, that when determining the ultimate validity of IgA/C3 ratio as a prognostic factor perhaps serum galactose-deficient IgA1 (Gd-IgA1) should be assessed, due to its essential role in the pathogenesis of IgAN, (Suzuki et al. 2011) rather than total IgA. This study was performed in a pediatric population of a single European country, which renders some informative value to the findings, but also constitutes a limitation.

5 Conclusions

1/ Predictors of poor outcome in childhood IgAN include the following: GFR reduction, nephrotic range proteinuria at onset of disease, and high MEST score in Oxford classification of kidney biopsy. 2/ Despite a higher serum IgA/C3 ratio in children with impaired renal function in long-term follow-up, we failed to demonstrate a significant association between this ratio at onset of disease and reduced GFR or persistent proteinuria at follow up. Thus, IgA/C3 ratio is not a good foreteller of progression of IgA nephropathy in childhood.

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

References

- Berger J, Hinglais N (1968) Inter-capillary deposits of IgA-IgG. *J Urol Nephrol* 74:694–695
- Coppo R, D'Amico G (2005) Factors predicting progression of IgA. *J Nephrol* 18:503–512
- Coppo R, Troyanov S, Bellur S, Cattran D, Cook HT, Feehally J, Roberts IS, Morando L, Camilla R, Tesar V, Lunberg S, Gesualdo L, Emma F, Rollino C, Amore A, Praga M, Feriozzi S, Segoloni G, Pani A, Cancarini G, Durlak M, Moggia E, Mazzucco G, Giannakakis C, Honsova E, Sundelin BB, Di Palma AM, Ferrario F, Gutierrez E, Asunis AM, Barratt J, Tardanico R, Perkowska-Ptasinska A, VALIGA study of the ERA-EDTA Immunonephrology Working Group (2014) Validation of the Oxford classification of IgA nephropathy in cohorts with different presentations and treatments. *Kidney Int* 86(4):828–836
- Komatsu H, Fujimoto S, Hara S, Sato Y, Yamada K, Eto T (2004) Relationship between serum IgA/C3 ratio and progression of IgA nephropathy. *Internal Med* 43:1023–1028
- Maeda A, Gohda T, Funabiki K, Horikoshi S, Shirato I, Tomino Y (2003) Significance of serum IgA levels and serum IgA/C3 ratio in diagnostic analysis of patients with IgA nephropathy. *J Clin Lab Anal* 17:73–76
- Mizerska-Wasiak M, Małydyk J, Rybi-Szumińska A, Wasilewska A, Miklaszewska M, Pietrzyk J, Firszt-Adamczyk A, Stankiewicz R, Bieniaś B, Zajaczkowska M, Gadomska-Prokop K, Grenda R, Pukajło-Marczyk A, Zwolińska D, Szczepańska M, Turczyn A, Roszkowska-Blaim M (2015) Relationship between serum IgA/C3 ratio and severity of histological lesions using the Oxford classification in

- children with IgA nephropathy. *Pediatr Nephrol* 30(7):1113–1120
- Roos A, Rastaldi MP, Calvaresi N, Oortwijn BD, Schlangwein N, Van Gijswijk- Janssen DJ, Stahl GL, Matsushita M, Fujita T, van Kooten C, Daha MR (2006) Glomerular activation of the lectin pathway of complement in IgA nephropathy is associated with more severe renal disease. *J Am Soc Nephrol* 17:1724–1734
- Schena FP (1990) A retrospective analysis of the natural history of primary IgA nephropathy worldwide. *Am J Med* 89:209–215
- Schwartz GJ, Muñoz A, Schneider MF, Mak RH, Kaskel F, Warady BA, Furth SL (2009) New equations to estimate GFR in children with CKD. *J Am Soc Nephrol* 20:629–637
- Suzuki H, Kiryluk K, Novak J, Moldoveanu Z, Herr AB, Renfrow MB, Eyatt RJ, Scolari F, Mestecky J, Gharavi AG, Julian BA (2011) The pathophysiology of IgA nephropathy. *J Am Soc Nephrol* 22:1795–1803
- Tesar V, Troyanov S, Bellur S, Verhave JC, Cook HT, Feehally J, Roberts IS, Cattran D, Coppo R, on behalf of the VALIGA study of the ERA-EDTA Immunonephrology Working Group (2015) Corticosteroids in IgA nephropathy: a retrospective analysis from the VALIGA Study. *J Am Soc Nephrol* 26(9):2248–2258
- Tomino Y (2003) Relationship between the serum IgA/C3 ratio and the progression of IgA nephropathy. *Intern Med* 43(11):1011
- Tomino Y, Suzuki S, Imai H (2000) Measurement of serum IgA and C3 may predict the diagnosis of patients with IgA nephropathy prior to renal biopsy. *J Clin Lab Anal* 14:220–223
- Working Group of the International IgA Nephropathy Network and the Renal Pathology Society, Roberts IS, Cook HT, Troyanov S, Alpers CE, Amore A, Barratt J, Berthouix F, Bonsib S, Bruijn JA, Cattran DC, Coppo R, D'Agati V, D'Amico G, Emancipator S, Emma F, Feehally J, Ferrario F, Fervenza FC, Florquin S, Fogo A, Geddes CC, Groene HJ, Haas M, Herzenberg AM, Hill PA, Hogg RJ, Hsu SI, Jennette JC, Joh K, Julian BA, Kawamura T, Lai FM, Li LS, Li PK, Liu ZH, Mackinnon B, Mezzano S, Schena FP, Tomino Y, Walker PD, Wang H, Weening JJ, Yoshikawa N, Zhang H (2009) The Oxford classification of IgA nephropathy: pathology definitions, correlations and reproducibility. *Kidney Int* 76:546–556
- Wyatt RJ, Kanayama Y, Julian BA, Negoro N, Sugimoto A, Hudson EC, Curd JG (1987) Complement activation in IgA nephropathy. *Kidney Int* 31:1019–1023
- Yoshikawa N, Tanaka R, Iijima K (2001) Pathophysiology and treatment of IgA nephropathy in children. *Pediatr Nephrol* 16:446–457

Index

A

Airway obstruction, 20, 21, 25
Antibiotics, 30, 40, 41, 43–45, 49, 60, 61

B

Bacteria identification, 40, 41
Bacterial strains, 3, 40, 44
Biomarker, 26, 51–56
Bordetella pertussis, 47–50

C

Cancer stage, 53, 56
Cancer tissue, 52
Candida spp., 1–7
Cardiac arrhythmia, 48, 49
Carriage, 2, 29–37
Chemical composition, 10, 11, 13, 14
Chronic cough, 49, 50
Chronic obstructive pulmonary disease (COPD), 1–7, 10, 17, 19–26, 61
Clostridium difficile, 59–62
COPD. *See* Chronic obstructive pulmonary disease (COPD)

D

Denture plaque, 1–7
Disease progression, 72
Drug susceptibility, 43
Dust, 11, 12

E

Exhaled breath condensate, 21–24

G

Genogrouping, 31–33
Genotyping, 3–7
Glycolysis, 52
Gram-negative bacteria, 5, 39–45

H

Health effects, 10

I

Induced sputum, 21, 23, 24
Infection, 2, 5–7, 20, 21, 30, 31, 33–37, 40, 41, 43–45, 47–50, 59–62
Intensive care, 30, 39–45, 61

K

Klebsiella spp., 1–7

L

Lung cancer, 51–56

M

Mortality risk, 59–62

N

Neisseria meningitidis, 29–37
Non-small cell lung cancer (NSCLC), 51–56
NSCLC. *See* Non-small cell lung cancer (NSCLC)

P

Particulate matter (PM), 9–17
Pneumonia, 2, 39–45, 59–62
Pneumonia prevalence, 40
Prevalence, 29–37, 40, 43–45
Prognosis, 52, 56, 66
Prognostic model, 51–56
Pyruvate kinase, 51–56

R

Respiratory tract, 10, 31, 33–35, 47, 48, 60

S

Salt aerosol, 10
Sensitivity, 22, 25, 40, 54–56, 67
Serogrouping, 31–33
Serum, 20–26, 33, 52–56, 60, 61, 66–69, 71, 72
Soldiers, 29–37, 44
Specificity, 25, 26, 54, 56, 67
Speleotherapy, 9

Subterraneotherapy, 9, 10, 17

Syncope, 48, 49

T

Thymic stromal lymphopoietin (TSLP), 19–26

V

Vaccination booster, 49, 50

W

Whooping cough, 47–50