Advances in Experimental Medicine and Biology 1021
Neuroscience and Respiration

Mieczyslaw Pokorski *Editor*

Pulmonary Care and Clinical Medicine

Advances in Experimental Medicine and Biology

Neuroscience and Respiration

Volume 1021

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Pulmonary Care and Clinical Medicine

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Opole, Poland

ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-3-319-65468-3 ISBN 978-3-319-65469-0 (eBook)
DOI 10.1007/978-3-319-65469-0

Library of Congress Control Number: 2017955089

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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 for organisms against pathogens and environmental or other sources of toxic or disease-causing effects. The articles provide timely overviews of contentious issues or recent advances in the diagnosis, classification, and treatment of the entire range of diseases and disorders, both acute and chronic. The texts are thought as a merger of basic and clinical research dealing with biomedicine at both the molecular and functional levels and with the interactive relationship between respiration and other neurobiological systems, such as cardiovascular function, immunogenicity, endocrinology and humoral regulation, and the mind-to-body connection. The authors focus on modern diagnostic techniques and leading-edge therapeutic concepts. Practical, data-driven options to manage patients are considered.

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

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

Concerning respiratory disorders, their societal and economic burden has been on the rise worldwide, leading to disabilities and shortening of life-span. COPD alone causes more than three million deaths globally each year. Concerted efforts are required to improve this situation, and part of those

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

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

Mieczyslaw Pokorski

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 33: 1–14
DOI 10.1007/5584_2017_25
© Springer International Publishing Switzerland 2017
Published online: 13 April 2017

Airway and Blood Inflammatory Markers in Waste Collectors

M. Raulf, V. van Kampen, H.D. Neumann, V. Liebers, A. Deckert, T. Brüning, J. Bünger, and F. Hoffmeyer

Abstract

Waste collectors are exposed to a heterogeneous mixture of bioaerosols able to induce health effects. The study aim was to evaluate inflammatory processes in blood and in the respiratory tract *via* analysis of atopy and club cell secretory protein 16 (CC16) in serum, exhaled nitric oxide (FeNO), and cellular and soluble mediators in nasal lavage fluid (NALF) and induced sputum (IS). Sixty nine current waste collectors (48% smokers) and 28 former waste collectors (25% smokers) were included in the cross-sectional study. In both groups, 63 and 64% of workers reported complaints of the eyes, nose and/or upper airways. Thirty two percent of the current and 25% of the former workers were classified as atopics. More atopics suffered from rhinitis and conjunctivitis than non-atopics (64% vs. 40% in current workers; 71% vs. 40% in former workers). CC16 values of present non-smokers were significantly higher compared to smokers. In total, FeNO values of 31 participants were lower than 10 ppb, 94% of them were smokers and 85% had respiratory symptoms of lower airways. Most of the IS biomarkers were significantly higher in smokers than in non-smokers. Non-smoking workers with respiratory symptoms of lower airways had slightly elevated mediator IS concentrations compared to asymptomatic non-smokers. We conclude that inflammatory changes in waste collectors are detectable in the content of IS biomarkers, exhaled NO, and serum CC16, which all are influenced by the smoking habit. No significant differences in biomarkers are detectable between current and former waste collectors.

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Keywords
Club cell • Exhaled nitric oxide • Induced sputum • Inflammatory markers • Nasal lavage • Non-invasive methods • Occupational exposure • Secretory protein • Waste collectors

1 Introduction

Professional collection of municipal solid waste is a task with heavy physical activities and exposure to a heterogeneous mixture of bioaerosols, gases, and vapours such as microbial and non-microbial volatile organic compounds (Wouters et al. 2002; Poulsen et al. 1995). These multiple work demands and hazards result in a higher incidence of health problems and injuries compared to other occupations (Velasco et al. 2015). In addition to occupational accidents and musculoskeletal problems, workers in the waste collection industry may have elevated incidence rates of work-related pulmonary, gastrointestinal, and skin problems compared to the entire work force (Poulsen et al. 1995). An important occupational hazard for waste collectors is exposure to bioaerosols because of the handling of organic material (Neumann 2014; Widmeier et al. 2007). Collection of household waste, separation, and composting generate organic dust which may contain high amounts of endotoxin and (1–3)β-D-glucan, depending on storage conditions (Neumann 2015). There is evidence that diseases caused by organic dust are mainly of inflammatory nature (Schantora et al. 2015; Raulf et al. 2015). Cross-sectional and cohort studies (van Kampen et al. 2012, 2016; Bünger et al. 2000, 2007) have shown that workers exposed to organic dust from composting plants had a higher prevalence of inflammatory upper airway and eye responses, the so-called mucous membrane irritation syndrome (MMIS). In addition, cases of hypersensitivity pneumonitis, organic dust toxic syndrome (ODTS), and allergic bronchopulmonary aspergillosis are reported (Bünger et al. 2007; Allmers et al. 2000). Non-invasive methods, such as the measurement of fractional exhaled nitric oxide (FeNO) and the collection and analysis of exhaled breath condensate (Hoffmeyer et al. 2014; Hoffmeyer et al. 2009), nasal lavage fluid (NALF) (Wouters et al. 2002), and induced sputum (IS) are useful in the assessment of inflammatory responses in the upper and lower respiratory tract in exposed workers (Raulf et al. 2015; Raulf-Heimsoth et al. 2011). Several studies have also demonstrated that exposure to organic dust components increases permeability of the bronchoalveolar epithelial barrier, causing leakage of pneumoproteins such as club cell secretory protein (CC16) or surfactant protein A (Daneshzadeh Tabrizi et al. 2010; Widmeier et al. 2007; Steiner et al. 2005). Thus, determination of these proteins in the bloodstream is a suitable option to assess inflammatory processes in the respiratory tract.

The objective of the present study was to evaluate inflammatory processes in blood and in the upper and lower respiratory tract in current and former waste collectors. Additional aspects of the study dealt with the correlation between clinical symptoms (e.g. rhinitis, chronic bronchitis, etc.) and the profile of inflammatory cellular and soluble markers in NALF and IS samples and with the evaluation of factors influencing the content of inflammatory markers.

2 Methods

The study design and protocol were created in accordance with the Declaration of Helsinki for Human Research. In May 2012, the Ethics committee of the Ruhr-University Medical School Bochum approved the implementation of all necessary examinations. All study participants gave written informed consent to participate in the study.

2.1 Study Group and Data Collection

In this cross-sectional study, 69 current waste collectors (mean weekly working time 39 h) were examined, representing six municipal solid waste management companies of the Ruhr area in North Rhine-Westphalia, Germany. In addition, 28 former waste collectors were also included in the study. The protocol and exposure circumstances of the study group were recently published (Hoffmeyer et al. 2016; Schantora et al. 2015; Neumann 2014, 2015). All current and former workers visited the Institute for Prevention and Occupational Medicine of the German Social Accident Insurance, Institute of the Ruhr-University Bochum (IPA) for clinical examination and data assessment. To ensure realistic measurements, all current workers were examined in an afternoon during a working week after their shift. All subjects were male. Smoking status was based on self-assessed information by interview and justified by cotinine concentrations in urine according to Xu et al. (2004). Study participants were classified as present, former, and never-smokers. For classification of the study group according to their clinical symptoms, such as mucus membrane irritation, cough, and chronic bronchitis, questionnaire data were used (Schantora et al. 2015). Diagnosis of COPD was based on FEV_1/FVC ratio below the lower limits of normal (LLN) as provided by the Global Lung Initiative (GLI) (Quanjer et al. 2012).

2.2 Measurements

Serological Parameters Serum of each worker was collected to determine total and specific immunoglobulin E (sIgE) in response to a variety of ubiquitous aero-allergens (atopy screen sx1 Phadiatop), and a mold mix (mx1) using the ImmunoCAP system of ThermoFisher Scientific/Phadia (Uppsala, Sweden). Allergen sIgE values $\geq$0.35 kU/L were considered positive and in the case of sx1 it was used to assess the atopy status of the workers. Additionally, concentration of CC16 secretory protein (former CC10, uteroglobin, or urinary protein 1) was also determined using a sandwich ELISA from BioVendor (Brno, Czech Republik) with a standard range of 1.57–50 ng/mL.

Nitric Oxide in Exhaled Breath FeNO was measured using a portable electrochemical analyser (NIOX Mino; Aerocrine, Solna, Sweden) according to the 2015 guidelines of the American Thoracic Society and European Respiratory Society (Hoffmeyer et al. 2016).

Nasal Lavage Fluid (NALF) and Induced Sputum (IS) NALF was collected by introducing a syringe with a suitable adapter with 7 mL of sterile physiological saline solution into a nostril, followed by its aspiration; the procedure repeated five times (Raulf et al. 2016; Raulf-Heimsoth et al. 2011). The residual fluid volume of NALF was recorded, centrifuged (5 min at 1300 rpm), the cell-free supernatant was divided into portions and frozen at −80 °C until further analysis of soluble biomarkers. The cell pellets were re-suspended and the total cell number was determined using the Neubauer counting chamber. For differential cell counts, slides were prepared by cytospin (Cytospin 2; Shandon Corp., Pittsburgh, PA) and stained with May-Grünwald-Giemsa. Two independent observers counted 200 cells on each slide under light microscopy. Their results were expressed as the percentage of a total cell number and the absolute cell count.

IS of each subject was collected after inhalation of isotonic (0.9%) saline aerosol, generated by an ultrasonic nebulizer for 10 min as described earlier (Raulf et al. 2015; Raulf-Heimsoth et al. 2011). The subjects were motivated to cough actively, clear their throat, and expectorate sputum. The IS volume was determined and a 2.5-fold quantity of 0.1% sputulysin (dithiothreitol) was added. The samples were gently vortexed and incubated for 30 min at 37 °C to ensure a complete homogenization. After centrifugation, cell-free supernatants were aliquoted, stored at

Table 1a Study group of 69 current waste collectors and 28 former waste collectors

	Current waste collectors	Former waste collectors
Male gender; n (%)	69 (100)	28 (100)
Age (year); median (range)	48 (23–63)	61.5 (38–72)
Employment (year); median (range)	22 (2–36)	25 (11–38)
Smoking status		
Present smokers; n (%)	33 (48)	7 (25)
Former smokers; n (%)	17 (24.5)	13 (46)
Never smokers; n (%)	19 (27.5)	8 (29)

Table 1b Clinical symptoms in current and former waste collectors

	Current waste collectors				Former waste collectors			
	Total (n = 69)	Never smokers (n = 19)	Former smokers (n = 17)	Present smokers (n = 33)	Total (n = 28)	Never smokers (n = 8)	Former smokers (n = 13)	Present smokers (n = 7)
Rhinitis; n (%)	27 (39)	7 (37)	11 (64)	9 (27)	13 (46)	3 (38)	5 (38)	5 (71)
Conjunctivitis; n (%)	20 (29)	9 (47)	6 (35)	5 (15)	6 (21)	2 (25)	2 (15)	2 (29)
Cough; n (%)	24 (35)	5 (26)	6 (35)	13 (39)	8 (29)	3 (38)	1 (8)	4 (57)
Chronic bronchitis; n (%)	11 (16)	1 (5)	4 (23)	6 (18)	5 (18)	1 (13)	0	4 (57)
COPD; n (%)	7 (10)	0	0	7 (21)	4 (14)	1 (13)	0	3 (42)

COPD chronic obstructive pulmonary disease

−80 °C under argon protection until further analysis of soluble markers. Cell pellets were further processed for the determination of the total cell number and for differential cytology, using the procedure described above for NALF.

The inflammatory mediators in NALF and IS were determined in the thawed cell-free supernatants. All samples underwent only a single freeze-thaw cycle. In both IS and NALF samples, the following soluble markers were measured: interleukin (IL)-8, total protein content, 8-isoprostane (8-iso-PGF$_{2\alpha}$), soluble (s) CD14, matrix metalloproteinase (MMP)-9, and tissue inhibitors of metalloproteinases (TIMP)-1. IL-8 was measured with the OptEIA™ ELISA (BD Biosciences Pharmingen; Heidelberg, Germany) in a standard range of 3–200 pg/mL. 8-isoprostane was quantified with a competitive immunoassay (Assay Design; Ann Arbor, CA) in a standard range of 6.1–100 ng/mL. Determinations of sCD14, TIMP-1, and MMP-9 were performed with the DuoSet™ ELISA Development system (R&D Systems; Wiesbaden Germany) in a standard range of 62.5–4,000 pg/mL for sCD14 and 31.2–2000 pg/mL for both MMP-9 and TIMP-1. Eosinophilic cationic protein (ECP) was quantified with the ImmunoCAP system from Thermo Fisher Scientific (Phadia AB, Uppsala, Sweden). Total protein content was determined according to the method of Bradford (1976) with bovine serum albumin as standard solution (range 10–100 μg/mL).

2.3 Statistical Analysis

Data were expressed as median with min-max range (tables) or interquartile range (figures). Data distribution was assessed using the D'Agostino and Pearson omnibus normality test. Values below the limit of quantification (LOQ) were set 2/3 of the LOQ. Comparison of unpaired data was performed with Mann-Whitney U or Kruskal-Wallis test and that of

Table 1c Duration of employment and symptoms from the *upper* and *lower* respiratory tract (RT)

	Current waste collectors (n = 69)			Former waste collectors (n = 28)		
Duration of employment	Number of employees; n (%)	Symptoms from upper RT; n (%)	Symptoms from lower RT; n (%)	Number of employees; n (%)	Symptoms from upper RT; n (%)	Symptoms from lower RT; n (%)
≤20 yr	28 (40%)	8 (28%)	13 (46%)	10 (36%)	6 (60%)	4 (40%)
	present non-smokers; 13	–	4 (30%)	present non-smokers; 4	–	1 (25%)
>20 yr	41 (60%)	5 (12%)	15 (37%)	18 (64%)	7 (39%)	2 (11%)
	present non-smokers; 13	–	7 (53%)	present non-smokers; 2	–	1 (50%)

Present non-smokers were both never smokers and former smokers

habits, e.g. smoking, between different groups with the Dunn multiple comparison test. A two-sided significance level of 0.05 was chosen for all tests. Data were analyzed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA).

3 Results

Table 1a presents the characteristics of the study group. The median age of the 69 current workers was 48 yr (range 23–63 yr) and median duration of employment was 22 yr (range 2–36 yr). The former waste collectors were older (median age 62 yr) at the time of examination with the median of 25 yr of employment (range 11–38 yr). Forty eight percent of current and 25% of former workers were present smokers. A high percentage (63% and 64%, respectively) of the workers reported complaints from the eyes, nose and/or upper airways (Table 1b). Ten percent of current waste workers had COPD and all of them were present smokers. Four out of the 28 (14%) of the former waste workers had COPD and one of them never smoked. A higher prevalence of current and former waste collectors with employment duration ≤20 yr reported symptoms from the upper respiratory tract (Table 1c). Although the percentage of current waste workers with symptoms from the lower respiratory tract was independent of employment duration, the percentage of present non-smokers (never and former smokers) in the group with longer employment was higher.

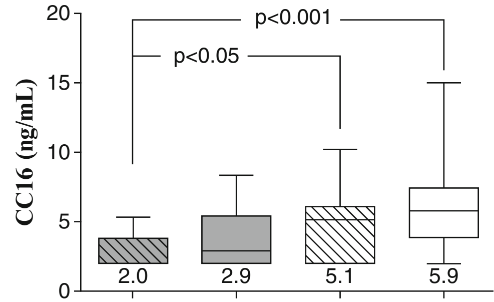
Data on the serum total and specific IgE and CC16 are summarized in Table 2. Elevated levels (>100 kU/L) of total IgE were present in 20 and 21% of current and former workers, respectively. Specific IgE values against the mold mixture were rare. Only 9% of current workers and 3.6% of former workers had a positive IgE response to the mold mixture. According to the sIgE-values in the inhalation atopy screening, 32% of current and 25% of former workers were classified as having atopy, without significant differences concerning smoking status. Rhinitis and/or conjunctivitis were more often reported in atopics than in non-atopics (64% vs. 40% in current workers and 71% vs. 40% in former workers). The serum CC16 concentration was significantly higher in never smokers than in present smokers in both current and former workers. In the current workers, former smokers also had a significant higher CC16 concentration than the present smokers. In the current workers, CC16 concentration was >2 ng/mL (limit of quantification) in just 52% of smokers, whereas CC16 exceeded that level in 84% of the never smokers. Similar differences were observed in the group of former waste collectors.

Since the serum CC16 concentration was not different between former and never smokers, both groups are combined for further CC16

Table 2 Serological results

	Current waste collectors				Former waste collectors			
	Total (n = 69)	Never smokers (n = 19)	Former smokers (n = 17)	Present smokers (n = 33)	Total (n = 28)	Never smokers (n = 8)	Former smokers (n = 13)	Present smokers (n = 7)
Total-IgE: kU/L; median (range)	46.7 (4.6–5,000)	30.6 (4.8–1,174)	39.6 (4.6–314)	54.4 (7.2–5,000)	46.3 (6.4–1,279)	47.1 (21.6–824)	34.2 (6.5–1,279)	50 (30.7–663)
Number of total-IgE >100 kU/L; n (%)	14 (20)	5 (26)	2 (12)	7 (21)	6 (21.4)	1 (12.5)	4 (31)	1 (14)
sIgE mold mix (mx1); number of sIgE ≥0.35 kU/L; n (%)	6 (9)	2 (11)	1 (6)	3 (9)	1 (3.6)	0	1 (7.7)	0
sIgE inhalation atopy-screen (sx1); number of sIgE ≥0.35 kU/L; n (%)	22 (32)	6 (31)	5 (29)	11 (33)	7 (25)	2 (25)	4 (31)	1 (14)
CC16: ng/mL; median (range)	4 (2–15)	5.9*[1] (2–15)	5.1*[2] (2–8.2)	2.4 (2–8.3)	4.3 (2–12.7)	6.4*[3] (2–10.1)	4.2 (2–12.7)	2.9 (2–4.3)
Number of CC16 > 2 ng/mL; n (%)	48 (70)	16 (84)	15 (88)	17 (52)	22 (79)	7 (88)	11 (85)	4 (57)

IgE immunoglobulin E, *CC16* club cell secretory protein 16
*[1]CC16 values between never smokers and present smokers were significantly different ($p < 0.01$)
*[2]CC16 values between former smokers and present smokers were significantly different in the group of current waste collectors ($p < 0.05$)
*[3]CC16 values between never smokers and present smokers were significantly different in the group of former waste workers ($p < 0.05$)



Symptoms from lower respiratory tract	+	−	+	−
Present smokers	+	+	−	−
Present non-smokers	−	−	+	+
Number of subjects	21	19	15	42

Fig. 1 Club cell protein (CC16) concentration in the study group ($n = 97$) stratified by smoking and symptoms from lower respiratory tract. $P < 0.001$ for differences among all four groups using Kruskal-Wallis test with Gaussian approximation and Dunn's multiple comparison test. Additionally, significant differences exist between present smokers with symptoms vs. present non-smokers with ($p < 0.05$) and without symptoms ($p < 0.001$)

Table 3 FeNO values of current and former waste collectors classified according their smoking habits

	Current waste collectors ($n = 69$)				Former waste collectors ($n = 28$)			
	n	Median (ppb)	Range (ppb)	Values <10 ppb (n)	n	Median (ppb)	Range (ppb)	Values <10 ppb (n)
Never smokers	19	15	9–137	1	8	14	10–38	0
Former smokers	17	20	6–109	1	13	16	12–40	0
Present smokers	33	8	1–33	24	7	9	4–15	5

ppb parts per billion

comparison with present non-smokers and smokers. Figure 1 shows the median CC16 concentration in present smokers and non-smokers, representing both current and former workers, with or without symptoms from the lower respiratory tract. The CC16 concentration of present smokers with symptoms was significantly lower compared with present non-smokers with ($p < 0.05$) or without ($p < 0.001$) symptoms. The CC16 concentration of present smokers without symptoms was not significantly different compared with present non-smokers with symptoms. There was no difference in CC16 concentration between present non-smokers with and without lower respiratory tract symptoms.

There were no significant differences in FeNO level between current and former workers (Table 3). The FeNO of 26 current workers was lower than 10 ppb, 92% of them were smokers and one half them had symptoms from the lower respiratory tract. Classification of all workers examined according to their smoking habits into present smokers and non-smokers, taking also into account the presence or absence of symptoms from the lower respiratory tract demonstrated that present smokers with or without symptoms had significantly lower FeNO values than the present non-smokers (Fig. 2). FeNO values of present non-smokers with or without symptoms were not different. Two non-smoking workers out of the 11 with symptoms from the lower respiratory tract had the FeNO exceeding 25 ppb.

The results of cellular and soluble biomarkers measured in NALF samples of current workers

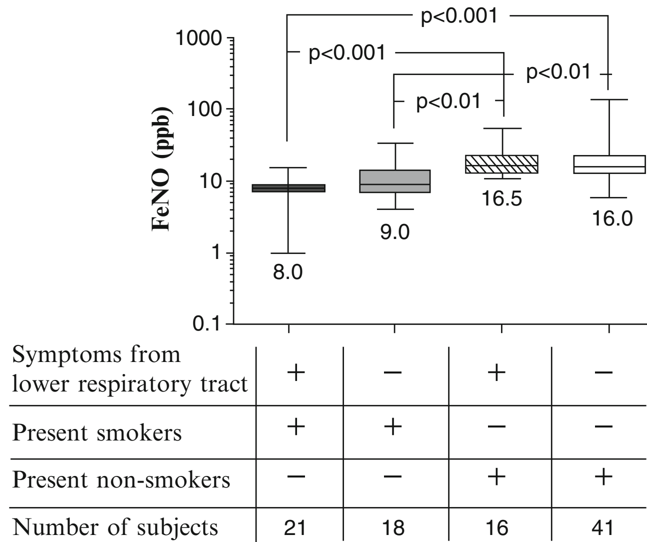


Fig. 2 Influence of smoking habits and symptoms from lower respiratory tract on exhaled nitric oxide content (FeNO). There were significant differences between present non-smokers, with or without symptoms, and present smokers ($p < 0.001$). No difference was observed between non-smokers with and without symptoms

Table 4a Cellular and soluble inflammatory markers in NALF in current waste collectors

	Current waste collectors			
	Total (n = 69); median (range)	Never smokers (n = 19); median (range)	Former smokers (n = 17); median (range)	Present smokers (n = 33); median (range)
Total cell count ($\times 10^4$)	8.4 (4.2–29.5)	8.4 (4.2–25.6)	10.6 (4.2–29.5)	8.4 (4.2–21.1)
Neutrophils (%)	7.5 (0–99.5)	22.5 (0–86.0)	7.0 (0–83.0)	7.0 (0–99.5)
IL-8 (pg/mL)	165 (3–2054)	238 (16–647)	94 (28–2054)	140 (3–1947)
MMP-9 (ng/mL)	4.1 (0.03–376)	14.8 (0.40–188)	2.5 (0.03–376)	2.8 (0.03–155)
TIMP-1 (ng/mL)	6.0 (0.03–97.2)	6.7 (0.13–24.8)	6.8 (0.42–45.8)	5.0 (0.03–97.2)
sCD14 (pg/mL)	799 (62.5–13,041)	1,051 (62.5–5,107)	802 (62.5–7,774)	515 (62.5–13,041)

are summarized in Table 4a and those of former workers are in Table 4b. There were no significant differences in the current waste collectors depending on the smoking habits, although the concentration of soluble biomarkers and the percentage of neutrophils tended to be lower in present smokers than in never smokers. The same trend was observed in former workers, although here two parameters reached significance, i.e., the percentage of neutrophils was significantly lower in present smokers than in never and former smokers and the sCD14-concentrations were significantly lower in present smokers than in former smokers. Comparison of NALF parameters between current and former waste collectors was inconspicuous. Classification of the subjects according to the presence of nasal symptoms (rhinitis yes or no) and their smoking habits showed no differences regarding the biomarkers in NALF (data not shown).

Table 5a summarizes the results of soluble biomarkers measured in IS samples of current waste collectors classified into never, former, and present smokers. The percentage of neutrophils, IL-8, TIMP-1, and total protein

Table 4b Cellular and soluble inflammatory markers in NALF in former waste collectors

	Former waste collectors				Statistics
	Total (n = 28); median (range)	Never smokers (=13); median (range)	Former smokers (n = 13); median (range)	Present smokers (n = 7); median (range)	Never (a) Former (b) Present (c)
Total cell count (×10^4)	8.3 (4.2–41.9)	8.5 (4.6–16.8)	4.2 (4.2–41.9)	4.2 (4.2–17.1)	–
Neutrophils (%)	9.5 (0–94)	30.7 (1.5–75)	47 (0.5–94)	0 (0–17.5)	a vs. c; $p < 0.05$ b vs. c; $p < 0.01$
IL-8 (pg/mL)	183 (3–9,079)	267 (36–9,079)	274 (94–3,014)	109 (3–591)	–
MMP-9 (ng/mL)	12.1 (0.03–380)	16.8 (0.6–137)	32.7 (3.4–380)	4.3 (0.03–43.7)	–
TIMP-1 (ng/mL)	6.8 (0.03–42.4)	4.9 (0.8–11.23)	11.3 (1.5–42.4)	4.8 (0.03–14)	–
sCD14 (pg/mL)	1,100 (62.5–5,862)	897 (94–4,704)	1,780 (143–5,862)	408 (62.5–2,164)	b vs. c; $p < 0.05$

IL-8 interleukin, *MMP-9* matrix metalloproteinase, *TIMP-1* tissue inhibitors of metalloproteinases, *sCD14* soluble sCD14

concentrations were significantly higher in smokers than in never or former smokers. Although all other IS parameters were higher in smokers than in never or former smokers, the differences did not reach statistical significance. A similar trend was observed in the former waste collectors (Table 5b), i.e., there were higher values in present smokers, but only the IL-8 level was significantly different between smokers and former smokers. In both groups, there were no significant differences in IS parameters between never and former smokers and, additionally, no differences between the current and former waste collectors. Therefore, to clarify the association between symptoms from the lower respiratory tract (cough, chronic bronchitis, or COPD) and inflammatory IS markers the study group was divided into four groups: present smokers with (A) and without (B) lower respiratory tract symptoms and present non-smokers with (C) and without (D) lower respiratory tract symptoms (Table 5c).

A significantly higher percentage of neutrophils ($p < 0.05$) was measured in IS samples of smoking subjects suffering from lower respiratory tract symptoms (group A) compared with non-smoking healthy subjects (group D). A similar pattern was detected for IL-8, i.e., significantly higher IL-8 concentration in both groups of smokers (groups A and C) and increasing, but not statistically different, IL-8 concentration between non-smokers with and without respiratory symptoms (group C vs. D). The level of the sputum immunoreactive MMP-9, TIMP-1, ECP, and sCD14 did not differ among the four groups. However, different concentrations of 8-isoprostane and total protein were found between groups B and D, both without clinical symptoms, with higher levels in smoking subjects (group B).

Table 5a Cellular and soluble inflammatory markers in induced sputum of current waste collectors

	Current waste collectors				Statistics
	Total (n = 69) median (range)	Never smokers (n = 19) median (range)	Former smokers (n = 17) median (range)	Present smokers (n = 33) median (range)	Never (a) Former (b) Present (c)
Total cell count ($\times 10^5$)	22.5 (4–99)	19.6 (5–49)	21 (6–78)	27.8 (5–99)	–
Neutrophils (%)	15.8 (0–61)	6.5 (1–38.5)	9.5 (0–61)	21.2 (1.5–59)	b vs. c; $p < 0.05$
Eosinophils (%)	0.5 (0–32)	0 (0–2)	0.5 (0–9.5)	1 (0–32)	–
IL-8 (pg/mL)	3,966 (3–84,546)	1,463 (3–6,139)	2,113 (402–25,102)	9,012 (3–84,546)	a vs. c; $p < 0.001$ b vs. c; $p < 0.05$
MMP-9 (ng/mL)	250 (0.03–1999)	150 (47–1,490)	220 (61–992)	284 (0.03–1999)	–
TIMP-1 (ng/mL)	48.6 (0.03–270)	25.0 (5.9–158)	64.0 (10.8–174)	58.0 (0.03–270)	a vs. c; $p < 0.05$
ECP (µg/mL)	25.3 (2–502)	21.9 (2–170)	19.0 (7–162)	37.7 (2–502)	–
8-isoprostane (pg/mL)	915 (6.1–11,713)	878 (71–2,809)	868 (6.1–7,278)	1,174 (175–11,713)	–
Total protein (µg/mL)	425 (63–2052)	276 (136–764)	367 (170–1,062)	512 (63–2052)	a vs. c; $p < 0.01$
sCD14 (pg/mL)	5,071 (178–30,599)	4,324 (1375–10,931)	3,781 (1832–17,392)	5,114 (178–30,599)	–

4 Discussion

Household waste collectors are exposed on a daily basis to significantly higher concentrations of bioaerosols than that present in the general living environment. In addition to our recently published data concerning health complaints of waste collectors (Hoffmeyer et al. 2016), here we studied the inflammatory propensity as evaluated from the content of serum total and specific IgE and CC16, and FeNO, and biomarkers in NALF and IS samples in current and former waste collectors. Since cigarette smoking is a well-known inducer of lung inflammatory processes, smoking habits were taken into account for all data analysis.

Specific IgE responses against the mold mix were rare in both current and former waste collectors and the number of subjects classified as atopics was nearly the same. The findings demonstrate that serum concentration of CC16, a sensitive biomarker of lung injury (Heldal et al. 2013), was not different between current and former waste collectors, but it was highly affected by smoking. CC16 is released by epithelial cells into the serum and provides an indication of acute exposure processes. Under chronic exposures causing tissue damage, CC16 concentration tends to be low. This is the case in chronic exposure to cigarette smoke, which damages club cells resulting in decreased serum CC16 (Hermans and Bernard 1999). The present findings demonstrate that the smoking current and former waste collectors had a significantly

Table 5b Cellular and soluble inflammatory markers in induced sputum of former waste collectors

	Former waste collectors				Statistics
	Total (=28) median (range)	Never smokers (n = 8) median (range)	Former smokers (n = 13) median (range)	Present smokers (n = 7) median (range)	Never (a) Former (b) Present (c)
Total cell count ($\times 10^5$)	22.0 (3–82)	15.5 (3–82)	22.3 (5–62)	32.0 (4.4–59)	–
Neutrophils (%)	14.0 (0.5–68.5)	10.7 (4–16)	10.5 (0.5–18.5)	46.0 (3–68.5)	–
Eosinophils (%)	0 (0–6.5)	0 (0–0)	0.25 (0–6.5)	1.5 (0–5.5)	–
IL-8 (pg/mL)	3,147 (12–146,992)	3,350 (572–8,697)	1,483 (12–5,686)	18,394 (3100–146,992)	b vs. c; $p < 0.05$
MMP-9 (ng/mL)	271.5 (8.4–4,699)	265 (105–865)	293 (8–4,699)	639 (56–1,408)	–
TIMP-1 (ng/mL)	47.2 (3.7–853)	42.8 (39–109)	50.0 (3.7–853)	186.0 (14.8–314)	–
ECP (μg/mL)	20.0 (2–218)	15.3 (10–64)	73.6 (10.7–218)	21.5 (2–91.4)	–
8-isoprostane (pg/mL)	921 (243–12,768)	1,810 (890–4,280)	600 (243–1,304)	1,135 (311–12,768)	–
Total protein (μg/mL)	403 (69–1,106)	293 (185–679)	364 (69–750)	715 (251–1,106)	–
sCD14 (pg/mL)	4,558 (399–12,267)	3,980 (1189–6,528)	5,737 (798–12,267)	3,619 (398–7,044)	–

lower CC16 concentration than never smokers. Additionally, CC16 concentration of present smokers with symptoms from the lower respiratory tract was significantly lower than that of present non-smokers with and without symptoms from the lower respiratory tract. No significant difference in CC16 content was found in waste collectors with or without symptoms from the lower respiratory tract. Norwegian workers exposed to sewage dust, containing a high content of a complex mix of microorganisms and their components, chemicals and gases, had a lower serum concentration of CC16 compared to the referents, also pointing to a long-term effect on secretion of this pneumoprotein (Heldal et al. 2013). That study has also shown that exposure to bacteria is associated with CC16 concentration, which may reflect a transient increased permeability of the lung-blood barrier. One reason for such results may be enhanced bioaerosol exposure, especially to endotoxin, in workers exposed to sewage dust (Heldal et al. 2016) compared to household waste collectors (Neumann 2014, 2015). Additionally, it should be taken into account that CC16 content is controlled by other factors, such as CC16 production by club cells, release into the alveolar space, renal clearance, and diffusion into the serum affected by pulmonary epithelial barrier permeability. Therefore, interpretation of CC16 content should be considered with caution (Lakind et al. 2007). A strong effect of smoking on the CC16 content also could be due to smoking-induced inhibition of activity of NO synthases (NOS), measurable as a lower level of fractional exhaled nitric oxide (FeNO) in smokers. In the present study, the majority of FeNO values were below 10 ppb in present smokers. The level of FeNO, as

Table 5c Lower respiratory tract symptoms (LRTS) and sputum parameters

	Present smokers with LRTS (n = 18) Group A; median (range)	Present smokers without LRTS (n = 18); Group B; median (range)	Present non-smokers with LRTS (n = 13) Group C; median (range)	Present non-smokers without LRTS (n = 34) Group D; median (range)	Statistics
Total cell count ($\times 10^5$)	31.9 (5.2–99.6)	27.8 (4.4–79)	15.5 (3.3–81.9)	22.2 (5.3–78)	–
Neutrophils (%)	23.3 (1.5–68.5)	18.3 (2.5–59.5)	10.5 (0–63.5)	8.75 (0.5–53)	A vs. D; $p < 0.05$
IL-8 (pg/mL)	12,146 (3–146,992)	11,538 (1666–90,492)	3,522 (3–56,862)	1,579 (12–25,102)	A vs. D; $p < 0.01$ B vs. C; $p < 0.05$ B vs. D; $p < 0.001$
MMP-9 (ng/mL)	269 (0.03–1999)	312 (56–1,361)	297 (81–4,699)	200 (8.5–1,491)	–
TIMP-1 (ng/mL)	58 (0.03–314)	58 (15–172)	63 (11–853)	40 (4–174)	–
ECP (μg/mL)	32 (2–502)	39 (9–190)	28 (10–170)	14 (2–115)	–
8-isoprostane (pg/mL)	811 (195–12,768)	2023 (175–11,713)	1,037 (6–4,280)	706 (70–7,278)	B vs. D; $p < 0.05$
Total protein (μg/mL)	561 (63–2052)	512 (233–1,314)	345 (185–860)	322 (69–1,274)	B vs. D; $p < 0.01$
sCD14 (pg/mL)	5,114 (178–30,599)	5,078 (1921–18,144)	4,009 (1189–12,267)	4,657 (799–17,392)	–

IL-8 interleukin, *MMP-9* matrix metalloproteinase, *TIMP-1* tissue inhibitors of metalloproteinases, *ECP* eosinophilic cationic protein, *sCD14* soluble sCD14

a biomarker of lower airway eosinophilic inflammation, was not different in present non-smokers, with and without clinical symptoms from the respiratory tract. Additionally, only two non-smokers with lower respiratory tract symptoms had FeNO above 25 ppb, indicating that eosinophilic Th2 driven (allergic) inflammation is not predominant in household waste collectors.

In this study we used a simple non-invasive technique of nasal lavage to evaluate inflammatory reactions in the upper airways. The findings demonstrate no appreciable differences in cellular and soluble markers of NALF between current or former workers or between workers with and without clinical symptoms from the upper airways. Conspicuously, however, content of biomarkers was lower in present smokers than that in non-smokers. In contrast to our results, which likely reflected subchronic effects of exposure to cigarette smoke, Heldal et al. (2003) have found an acute increase in NALF biomarkers in waste handlers, associated with neutrophil infiltration, after three working days from Monday to Thursday after a weekend break. Those authors have not observed any cigarette smoking dependencies.

In the present study we detected pronounced inflammatory effects concerning the cellular and soluble sputum biomarkers. There were apparent differences between present and never or former smokers. Akin to previous studies conducted in composting plant workers (Raulf et al. 2015) and bitumen exposed workers (Raulf-Heimsoth et al. 2011), immunoreactive MMP-9 concentration in sputum was higher in smokers than in present

non-smokers. We failed to confirm signs of remission of a subchronic inflammatory state in workers exposed to bioaerosols once exposure is terminated, observed in our previous study in composting plant workers (Raulf et al. 2015) and in a study of Sikkeland et al. (2012) in workers exposed to organic dust. Some sputum biomarkers such as neutrophils, IL-8, MMP-9, TIMP-1, and 8-isoprostane, were higher in former than never smoking waste collectors, which however failed to reach statistical significance. Taking lower airway symptoms into account, as expected, smoking caused greater effects on the biomarker content. Although IL-8, MMP-9, TIMP-1, and 8-isoprostane concentrations tended to be higher in non-smokers with lower airway symptoms compared with non-smokers without the symptoms, no significant difference were detectable.

The cross-sectional design of the present study, without a control group, is a weakness. Longitudinal studies consisting of a non-invasive assessment of inflammatory biomarkers should be performed in waste collectors to examine the possible causal relationship between working environment, exposure, and respiratory health problems. However, a strength of the present study is that all measurements were done with standardized, validated procedures in 69 current waste collectors and 28 former waste collectors, and the potential confounding factors such as smoking, age, and atopy were taken into consideration. Our results also provide an indication that allergic Th2-triggered inflammation was not the primary health problem in the workers with a median employment of 22 yr. This may be a result of a not more explained "healthy worker effect." Although the inflammatory effects measured were not statistically different between non-smokers with or without respiratory symptoms, implementation of the biomarker measurement using different matrices is useful to assess the airway condition in waste collectors exposed to bioaerosols. In this population of blue-color workers anti-smoking and dust reduction programs should be implemented to avoid airway inflammation and respiratory health problems.

Acknowledgment The study was conducted with the help of the German Social Accident Insurance, Institution for the public sector in North Rhine-Westphalia, Düsseldorf, Germany. We would like to thank the waste collectors for participating in the study. We gratefully acknowledge the support of the laboratory and clinical staff for their skilful technical assistance. Supported in part by the German Social Accident Insurance (project IPA-94).

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

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 33: 15–26
DOI 10.1007/5584_2017_23
© Springer International Publishing Switzerland 2017
Published online: 6 April 2017

Malnutrition and Quality of Life in Patients with Non-Small-Cell Lung Cancer

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Abstract

Progressive weight loss, common reduces performance and quality of life in patients with advanced lung cancer. However, there is a paucity of studies that focus on nutritional status and quality of life of non-small cell lung cancer (NSCLC) patients. The present study seeks to determine the nutritional status, and its relation to quality of life, of NSCLC patients. One hundred and eighty NSCLC patients (mean age 62.8 ± 9.6 years) were evaluated during therapy at the Lower Silesian Center of Lung Diseases in Wroclaw, Poland. Nutritional status was evaluated by means of the Mini-Nutritional Assessment (MNA) and quality of life by means of two instruments developed by the European Organization for the Research and Treatment of Cancer (EORTC): QLQ-C30 and QLQ-LC13 questionnaires. The MNA revealed that up to 51.1% of patients were undernourished, 23.9% were at risk of malnutrition, and only 25.0% showed a normal nutrition. The well-nourished respondents evaluated their quality of life better in all functional scales (33.3 *vs.* 41.7 *vs.* 66.7, respectively) and presented less intensive symptoms in general

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QLQ-C30 and specific LC13 questionnaires. In univariate analysis, malnutrition significantly correlated with decreased quality of life and the intensity of symptoms in both questionnaires. In multivariate analysis, malnutrition was an independent determinant of decreased quality of life in physical functioning domain ($\beta = -0.015$; $p < 0.001$). We conclude that malnutrition has an impact on quality of life and on the presentation of symptoms in NSCLC patients. Therefore, nutritional care should be integrated into the global oncology as an adjunct to symptomatic treatment.

Keywords

Quality of life • Malnutrition, Mini-nutritional assessment • Non-small cell lung cancer • Nutrition

1 Introduction

Lung cancer is the leading cause of cancer death among men and women worldwide. A vast majority of patients suffer from an advanced form of the disease at diagnosis. Despite the use of new therapeutic agents and modest survival improvement, overall prognosis for lung cancer patients is unfavorable (Plunkett et al. 2003). Lung cancer is a tumor associated with a relatively high incidence of malnutrition, sarcopenia, and cachexia, which cause physical functioning disorders, lower quality of life, and increased resistance to treatment (Kovarik et al. 2014). Progressing cancer negatively affects general condition of patients, including their nutritional status. Deteriorating nutrition may, in turn, decrease response to treatment, enhance side effects of therapy, and shorten survival time. Several reports show that protein energy malnutrition (PEM) may occur in about 15–40% of patients at diagnosis and in 50–80% of those with advanced cancer, depending on the tumor type, its location, stage, and treatment (Bauer et al. 2002).

Cancer cachexia is a complex condition. The pathogenesis of cachexia involves numerous factors such as oral food intake disorders, increased nutrient loss, metabolic disorders, enhanced inflammation induced by inflammatory cytokines, enhanced catabolic processes due to circulating factors, increased demand for food due to tumor or concomitant diseases, increased energy output, and adverse effects of cancer therapy (Aapro et al. 2014). Negative effects of malnutrition include progressive weight loss caused by fat and muscle tissue loss, weakness, fatigue, metabolic and immune system disorders, and infectious complications. In 5–20% of patients, cachexia constitutes the immediate cause of death in end-stage cancer. According to experts, patients most prone to cachexia are children and the elderly, as well as patients with gastrointestinal, head and neck, lung and prostate cancer. Symptom intensity is often proportional to the severity of the disease. Also, treatment comprising chemotherapy, radiotherapy, and surgical regimens is associated with acute and chronic symptoms, which might cause or aggravate nutritional disorders (Shahmoradi et al. 2009).

Studies indicate that good nutritional status results in better quality of life in cancer patients. The American Society for Parenteral and Enteral Nutrition (ASPEN) recommends nutritional screening, nutritional assessment and intervention for cancer patients. Correcting malnutrition may improve quality of life in these patients, which is an important outcome for patients themselves, their caregivers, and families (Lis et al. 2012). Nutritional status assessment performed

when diagnosing the patient allows for early medical intervention to prevent cancer cachexia. The tools used to assess nutritional status of the patient include anthropometric measurements such as weight change, arm muscle circumference, and triceps skin-fold thickness, biochemical parameters such as serum albumin, and standardized questionnaires (Trabal et al. 2006).

Nutritional risk screening is obligatory in all hospital wards in Poland. The following questionnaires are used on admission: Subjective Global Assessment or Nutritional Risk Screening. These questionnaires should be administered every 14 days. Cancer patient quality of life is a subjective, multi-dimensional assessment concerning five functional dimensions (i.e., physical, role, emotional, cognitive, and social), severity of symptoms, financial problems, and the global quality of life (Polanski et al. 2016; Ravasco et al. 2003). Quality of life in lung cancer patients is lower compared to healthy population, but also lower compared to patients suffering from other types of cancer. In the case of lung cancer, quality of life is determined by the severity and the number of symptoms such as fatigue, loss of appetite, dyspnea, cough, pain, and blood in sputum, which are specific for lung tumors. Fatigue and respiratory problems diminish quality of life in psychological dimension, while sleep problems reduce cognitive functioning. Physical dimension, related to growing disability, decreases in most patients, which makes them unable to play family and social roles. The disease is a frequent reason of irritation, distress, and depression (Polanski et al. 2016). The literature most often focuses on questions regarding quality of life in relation to non-small cell lung cancer (NSCLC) treatment, but there are few studies on the relationship between nutritional status and quality of life in lung cancer patients. Therefore, the aim of this study was to assess the influence of nutritional status on quality of life in NSCLC patients.

2 Methods

2.1 Patients

The study was approved by the Ethics Committee of Wroclaw Medical University in Poland (approval no. KB-507/2015). It was conducted between January and December 2015 in the Lower Silesian Center for Lung Diseases in the city of Wroclaw. There were 180 patients of the mean age of 62.8 ± 9.6 years enrolled into the study who were qualified by an oncologist. All patients provided written informed consent to participate and completed the questionnaires in the presence of a clinician. The inclusion criteria were the following: lung cancer diagnosis confirmed by a histopathological examination, age greater than 18 years, and comprehending the questionnaire's items. The exclusion criteria were the following: uncertain cancer diagnosis, lack of consent to participate in the study, coexistence of other severe chronic diseases that could influence the patient's perception of health status such as other malignant tumors, heart insufficiency aggravation, severe chronic obstructive pulmonary disease, asthmatic condition, or hemodynamic instability; and cognitive impairments. The patients were divided into three groups depending on the Mini Nutritional Assessment (MNA) score: Group 1 – malnourished (n = 92; 51.1%), Group 2 at risk of malnutrition (n = 43; 23.9%), and Group 3 normal nutritional status (n = 45; 25.0%).

2.2 Questionnaire Methodology

All patients were surveyed and their socio-demographic and clinical data were gathered from medical records. We used standardized questionnaire tools for quality of life assessment developed by the European Organization for the Research and Treatment of Cancer (EORTC). They were the following: the core quality of life

questionnaire QLQ C-30, covering general aspects of health-related quality of life, supplemented by the 13-item module LC-13, covering lung cancer-specific health-issues. The QLQ-C30 evaluates self-assessed health status and social, physical, and emotional functioning of cancer patients. It consists of 30 items, grouped into 5 functional scales: physical functioning (5 questions), role functioning (2 questions), emotional functioning (4 questions), cognitive functioning (2 questions), and social functioning (2 questions). It also includes 3 symptom scales: fatigue (3 questions), nausea and vomiting (2 questions), and pain (2 questions), as well as 6 single questions evaluating the severity of the following symptoms: dyspnea, insomnia, appetite loss, constipation, diarrhea, and financial difficulties. The last two questions pertain to global health status assessment. Respondents provide answers to the questions using a 4-grade intensity scale: never (1), sometimes (2), often (3), and very often (4). The raw scores are linearly transformed to give standard scores in the range of 0–100 for each of the functional and symptom scales. Higher scores in the global and functional scales and lower scores in the symptom scales indicate better quality of life. The QLQ-LC13 questionnaire contains 13 questions concerning typical lung cancer symptoms.

Performance status was assessed in cancer patients using one the most popular scoring systems, i.e., the Eastern Cooperative Oncology Group scale, (ECOG/WHO/Zubrod scale), published by Oken et al. (1982) which runs from 0 to 5, with 0 denoting perfect health and 5 death.

The Mini Nutritional Assessment (MNA) questionnaire consists of two parts: short initial screening, i.e., MNA-Short Form (MNA-SF) and its continuation (the full MNA). What is assessed in the first part of the questionnaire is decline in food intake, weight loss, and occurrence of psychological stress or acute disease in the three months before screening, as well as body mass index (BMI) and patients' mobility. Maximum score in this part is 14 points. The second part of the questionnaire, i.e., patients' assessment, provides information on the number of meals, mode of feeding, components of the diet, and drugs prescribed. It also includes the measurement of mid-arm and calf circumferences. In this part, the maximum score is 16 points. The full MNA score is a sum of points from the first and second part of the questionnaire. The total maximum score is 30 points. Three categories of nutritional status are proposed: normal nutritional status 24–30 points, at risk of malnutrition 17–23.5 points, and malnourished <17 points. Validation studies demonstrate a high reliability and accuracy of this tool (sensitivity – 97.9%, specificity – 100%) (Rubenstein et al. 2001).

Data on the time of lung cancer diagnosis, treatment administered, tumor type, TNM classification, spirometry results, genetic predisposition to cancer, tobacco smoking, and other essential socio-demographic information were gathered from medical records.

2.3 Statistical Elaboration

In the tables presenting basic descriptive statistics for quantitative variables, mean values $\pm$SD were given when the Kolmogorov-Smirnov tests confirmed normality of the empirical distribution at $p = 0.05$. If the hypothesis on normal distribution was rejected, the median values with the difference between the upper and lower quartiles ($Q_1 - Q_s$) were given. The hypothesis on the lack of differences in quantitative variables in the subgroups analyzed was verified using Student's *t*-test, unless variable distribution in both groups significantly deviated from normal distribution. If a variable in at least one of the groups did not follow a normal distribution, the Mann–Whitney U test was used. Contingency tables were created to display ordinal and categorical variables. Then, verification of the hypothesis on the independence assumption was conducted using Pearson's x^2 test.

3 Results

3.1 Patient Characteristics

Table 1 presents the socio-demographic characteristics of the study patients. More than half of them (58.9%) were married or had a partner. A large majority (67.6%) were benefit claimants or retirees. These were mostly people with vocational (48.6%) or high-school education (33%). About a third (37.8%) of patients were genetically predisposed to the disease due to family history of cancer.

The TNM Classification of Malignant Tumors showed that T2 (41.1%) and N2 (spread to ipsilateral lymph nodes of the mediastinum) (28.8%) were diagnosed most often. As for the presence of distant metastasis, M0 (no distant metastasis) was most frequently observed (69.2%) (Table 2).

Lung cancer was most frequently diagnosed by a family physician (68.6%), distinctly less frequently by a specialist physician (19.0%). Predominating symptoms experienced by patients were: chronic cough (80.5%), dyspnea (61.6%), chest pain (40.0%), hemoptysis (30.8%), and recurring infections (28.1%). The patients were diagnosed with the following comorbidities: diabetes (29.2%), asthma or chronic obstructive pulmonary disease (20.5%), heart failure (19.5%), and ischemic heart disease (15.1%). Sixty five percent of patients believed the tumor had not spread to other organs. Despite suffering from lung cancer, 37.3% of the patients continued to smoke (Table 3).

Spirometry revealed diminished FEV1 – 2.37 ± 0.80 L, FVC – 3.06 ± 0.96 L, and

Table 1 Socio-demographic characteristics of non-small-cell lung cancer (NSCLC) patients (n = 180)

Age (years)	
Mean ± SD	62.8 ± 9.6
Median ($Q_1 - Q_3$)	63 (58–68)
Min – Max	25–87
Number of hospitalizations:	
Mean ± SD	1.0 ± 1.7
Median ($Q_1 - Q_3$)	0 (0–1)
Min – Max	0–11
	n (%)
Residence	
City/town	134 (72.4)
Village	51 (27.6)
Source of income	
Permanent job	54 (29.2)
Unemployment benefit/disability pension/retirement pension	125 (67.6)
Family support	6 (3.2)
Marital status	
Married/in a relationship	109 (58.9)
Single/widower/widow	76 (41.1)
Education	
Primary	15 (8.1)
Vocational	90 (48.6)
Secondary	61 (33.0)
Higher	19 (10.3)
Family history of cancer	
Yes	70 (37.8)
No	115 (62.2)

Table 2 TNM classification of non-small-cell lung cancer (NSCLC) patients (n = 180)

	n (%)
T1	41 (22.2)
T2	76 (41.1)
T3	21 (11.4)
T4	46 (24.9)
Tx	2 (1.1)
N0	68 (36.8)
N1	42 (22.7)
N2	52 (28.1)
N3	11 (5.9)
Nx	11 (5.9)
M0	128 (69.2)
M1	34 (18.4)
M2	4 (2.2)
M3	1 (0.5)
Mx	17 (9.2)

FEV1/FVC – 79.4 ± 20.6% in the patients studied. As for the functional status assessed with the Zubrod scale, patients usually scored 1 or 2 (37.5% and 36.1%, respectively), which indicates difficulties in carrying out housework and job tasks. The patients underwent the following types of treatment: surgery (71.4%), radiotherapy (29.7%), and chemotherapy (55.1%) (Table 4).

3.2 Nutritional Status and Quality of Life – Comparative Analysis

Details of the quality of life assessment with EORTC QLQ-C 30 and LC 13 questionnaires in relation to the patients' nutritional status assessed with MNA are presented in Table 5. The following relations were unraveled:

- patients whose nutritional status was normal had a significantly better global quality of life compared to the other patients;
- patients whose nutritional status was normal and those at risk of malnutrition displayed significantly better physical functioning compared to malnourished patients;
- patients whose nutritional status was normal displayed significantly better role and cognitive functioning compared to patients at risk of malnutrition who, in turn, were

Table 3 Clinical characteristics of non-small-cell lung cancer (NSCLC) patients (n = 180) continued

	n (%)
X-ray commissioned by	
Family physician	127 (68.6)
Patient himself/herself	10 (5.4)
Family advice	16 (8.6)
Specialist physician	36 (19.5)
Other	1 (0.5)
Tumor diagnosed with	
CXR	106 (57.3)
CT	116 (62.7)
Bronchoscopy	55 (29.7)
Sputum cytology	23 (12.4)
Biopsy	77 (41.6)
Smoking	
Yes	69 (37.3)
No, never	36 (19.5)
Not any more	71 (38.4)
No, but my family smokes	9 (4.9)
Chronic diseases	
Diabetes	54 (29.2)
IHD	28 (15.1)
Renal failure	6 (3.2)
RA	4 (2.2)
Heart failure	36 (19.5)
Asthma/COPD	38 (20.5)
Metastasis	
None	121 (65.4)
Bone tissue	10 (5.4)
Brain	8 (4.3)
Liver	17 (9.2)
Adrenal gland	20 (10.8)
Multiple organs	16 (8.6)
Symptoms	
Chronic cough	149 (80.5)
Dyspnea	114 (61.6)
Pain in chest	74 (40.0)
Hemoptysis	57 (30.8)
Recurring infections	52 (28.1)
SVCS	4 (2.2)
Cardiac dysrhythmia	7 (3.8)
Dysphonia	52 (28.1)

CXR chest X-ray, *CT* computed tomography, *IHD* ischemic heart disease, *RA* rheumatoid arthritis, *COPD* chronic obstructive pulmonary disease, *SVCS* superior vena cava syndrome

Table 4 Spirometry results and Zubrod score in non-small-cell lung cancer (NSCLC) patients (n = 180)

FEV1 (L)	
Mean ± SD	2.4 ± 0.8
Median ($Q_1 - Q_3$)	2.2 (1.8–2.8)
Min – Max	0.8–4.4
FVC (L)	
Mean ±SD	3.1 ± 1.0
Median ($Q_1 - Q_3$)	2.9 (2.3–3.7)
Min – Max	1.0–6.3
FEV1/FVC (%)	
Mean± SD	79.4 ± 20.6
Median ($Q_1 - Q_3$)	77.1 (69–86)
Min – Max	22.9–160.6
Zubrod score	***n (%)***
0 – asymptomatic, fully active	34 (18.1)
1 – symptomatic, reduced activity, able to carry out light work	80 (37.5)
2 – capable of all self-care, unable to carry out any work activities	61 (36.1)
3 – capable of limited self-care	9 (4.2)
4 – incapable of any self-care	1 (0.5)

significantly better in this respect compared to malnourished patients;

- patients whose nutritional status was normal displayed significantly better emotional and social functioning compared to the other patients;
- patients whose nutritional status was normal were significantly less affected by fatigue, pain, insomnia, dyspnea and financial difficulties compared to patients at risk of malnutrition who, in turn, were significantly less affected than malnourished patients;
- patients whose nutritional status was normal and those at risk of malnutrition were significantly less affected by nausea and vomiting, appetite loss, diarrhea and cough compared to malnourished patients;
- patients whose nutritional status was normal were significantly less affected by dyspnea, sore mouth or tongue, pain in chest, and pain in arm or shoulder compared to malnourished patients;
- patients whose nutritional status was normal were significantly less affected by troubles with swallowing, peripheral neuropathy and pain in other parts of the body compared to the other patients.

3.3 Relationships Correlation Among Nutritional Status, Quality of Life, and Symptom Severity

As for nutritional status and quality of life, we observed an inverse correlation with the functional scales of EORTC QLQ-C30, which means that the more severe malnutrition, the lower was quality of life. There also was a positive correlation between nutritional status and the symptom scales, which means that the more severe malnutrition, the higher was severity of symptoms within all domains of the EORTC questionnaire (Table 6).

The multivariate analysis revealed that nutritional status (malnutrition index) is an independent determinant of diminishing quality of life within the physical functioning scale ($\beta = -0.17$; $p = 0.001$), and of increasing severity of nausea and vomiting ($\beta = 0.005$, $p = 0.009$) and insomnia ($\beta = 0.003$, $p = 0.011$) within the symptom scales (Table 7).

Table 5 Quality of life assessment with EORTC QLQ-C 30 and LC 13 depending on the patients' nutritional status

Scale		Malnourished		At risk of malnutrition		Normal nutritional status		Statistics*
		Median	$Q_1 - Q_3$	*Median*	$Q_1 - Q_3$	*Median*	$Q_1 - Q_3$	
QLQ-C30 functional	Global quality of life	33.3	25.0–50.0	41.7	29.2–58.3	66.7	66.7–83.3	$p < 0.001$
	Physical functioning	66.7	53.3–73.3	80.0	66.7–86.7	86.7	80.0–93.3	$p < 0.001$
	Role functioning	66.7	33.3–66.7	66.7	50.0–100	100	66.7–100	$p < 0.001$
	Emotional functioning	41.7	33.3–66.7	50.0	33.3–83.3	91.7	75–100	$p < 0.001$
	Cognitive functioning	66.7	50.0–83.3	83.3	66.7–100	100	83.3–100	$p < 0.001$
	Social functioning	50.0	33.3–83.3	66.7	33.3–100	100	83.3–100	$p < 0.001$
QLQ-C30 symptom	Fatigue	55.6	33.3–66.7	44.4	22.2–55.6	11.1	11.1–44.4	$p < 0.001$
	Nausea and vomiting	16.7	0–33.3	0	0–16.7	0	0–0	$p < 0.001$
	Pain	33.3	33.3–66.7	33.3	16.7–50.0	16.7	0–33.3	$p < 0.001$
	Dyspnea	33.3	33.3–66.7	33.3	33.3–66.7	33.3	33.3–33.3	$p = 0.001$
	Insomnia	66.7	33.3–66.7	33.3	0–66.7	0	0–33.3	$p < 0.001$
	Appetite loss	33.3	33.3–66.7	33.3	0–33.3	0	0–33.3	$p < 0.001$
	Constipation	0	0–33.3	0	0–33.3	0	0–33.3	$p = 0.13$
	Diarrhea	0	0–33.3	0	0–0	0	0–0	$p = 0.006$
	Financial difficulties	66.7	33.3–66.7	33.3	0–33.3	0	0–0	$p < 0.001$
QLQ-LC13	Dyspnea	33.3	33.3–66.7	22.2	11.1–33.3	11.1	11.1–22.2	$p < 0.001$
	Cough	66.7	33.3–66.7	33.3	33.3–66.7	33.3	33.3–33.3	$p < 0.001$
	Hemoptysis	0	0–33.3	0	0–33.3	0	0–33.3	$p = 0.315$
	Sore mouth or tongue	0	0–33.3	0	0–0	0	0–0	$p = 0.016$
	Trouble swallowing	0	0–33.3	0	0–33.3	0	0–0	$p < 0.001$
	Peripheral neuropathy	0	0–33.3	0	0–33.3	0	0–0	$p = 0.011$
	Alopecia	0	0–33.3	0	0–0	0	0–0	$p = 0.084$
	Chest pain	33.3	0–33.3	0	0–33.3	0	0–33.3	$p < 0.001$
	Arm or shoulder pain	0	0–33.3	0	0–33.3	0	0–0	$p = 0.045$
	Pain in other parts	33.3	0–66.7	33.3	0–33.3	0	0–0	$p < 0.001$
	Pain medication	75.0	50.0–75.0	50.0	0–75.0	50.0	0–75.0	$p = 0.524$

*Kruskal–Wallis test

4 Discussion

Self-assessed quality of life is a highly significant outcome measure for cancer patients. The physical and psychological conditions of patients determine their approach toward the disease and strength they exert to fight it away. Patients' well-being has an enormous influence on daily functioning and the ability to perform social and work activities.

Cancer and its treatment affect nutritional status by changing metabolism and decreasing food intake. Studies demonstrate that malnutrition is a

Table 6 Relationships between EORTC QLQ C-30 and LC-13 domains, and nutritional status evaluated with Mini Nutritional Assessment (MNA) in non-small cell lung cancer (NSCLC) patients

	Nutritional status Malnutrition index	
Variable	*r*	p
EORTC QLQ-C30		
Global health status	−0.450	<0.001
Physical functioning	−0.536	<0.001
Role functioning	−0.474	<0.001
Emotional functioning	−0.328	<0.001
Cognitive functioning	−0.414	<0.001
Social functioning	−0.256	<0.001
Fatigue	0.470	<0.001
Nausea and vomiting	0.317	<0.001
Pain	0.384	<0.001
Dyspnea	0.294	<0.001
Insomnia	0.373	<0.001
Appetite loss	0.403	<0.001
Constipation	0.266	<0.001
Diarrhea	0.152	0.040
Financial difficulties	0.341	<0.001
EORTC QLQ-LC-13		
Dyspnea	0.374	<0.001
Cough	0.421	<0.001
Pain in chest	0.255	<0.001
Pain in other parts	0.214	<0.001

predictor of morbidity in advanced cancer and is an important factor in predicting risk associated with surgery for cancer patients. Hence, malnutrition is liable to assume a significant role in patients' quality of life (Lis et al. 2012; Scott et al. 2003).

According to the National Cancer Institute's Nutrition in Cancer Care guidelines, timely identification and treatment of nutrition problems may improve cancer patient prognosis by helping them gain or maintain weight, improving their response to therapy, and reducing treatment complications (Gupta et al. 2006). Ravasco et al. (2004) have demonstrated in a group of cancer patients that their weight loss is related to the stage of cancer, and is greater in stages III/IV than in I/II. Those authors also have found that the contribution of nutritional status to quality of life is 20%, and of weight loss and tumor location is 30% each in cancer patients. Quality of life in lung cancer patients is distinctly lower compared to healthy population (Handy 2011; Lemonnier et al. 2011).

According to some authors, low socioeconomic status, limited functioning, and a greater severity of symptoms are significant determinants of diminished quality of life in lung cancer patients (Zimmermann et al. 2011 Montazeri et al. 2001; Mor et al. 1994). In the present study, a considerable number of patients did not work, received sickness benefits, and were single, which usually is related to lower economic status.

Iyer et al. (2013), assessing symptom severity with Lung Cancer Symptoms Scale (LCSS), have confirmed that the most frequent lung cancer symptoms are: fatigue (98% of patients), appetite loss (98%), shortness of breath (94%), cough (93%), pain (90%), and blood in sputum (70%). Those authors also have demonstrated that a higher severity of symptoms was accompanied by lower quality of life, which is particularly affected by appetite loss, fatigue, pain, and dyspnea. Other studies also have shown that the more symptoms of the disease, the lower quality of life (Hermann and Looney 2011; Henoch et al.

Table 7 Regression coefficients between selected variables and nutritional status (MNA – malnutrition index) in in non-small cell lung cancer (NSCLC) patients (n = 180) in univariate and multivariate analysis

Variable	Univariate		Multivariate	
	β	p	β	p
EORTC QLQ-C30				
Global health status	−0.014	<0.001	0	NS
Physical functioning	−0.021	<0.001	−0.015	<0.001
Role functioning	−0.012	<0.001	0	NS
Emotional functioning	−0.008	<0.001	0	NS
Cognitive functioning	−0.014	<0.001	0	NS
Social functioning	−0.006	<0.001	0	NS
Fatigue	0.012	<0.001	0	NS
Nausea and vomiting	0.012	<0.001	0.005	0.009
Pain	0.011	<0.001	0	NS
Dyspnea	0.008	<0.001	0	NS
Insomnia	0.008	<0.001	0.003	0.011
Appetite loss	0.010	<0.001	0	NS
Constipation	0.007	<0.001	0	NS
Diarrhea	0.005	0.040	0	NS
Financial difficulties	0.008	<0.001	0	NS
EORTC QLQ-LC-13				
Dyspnea	0.118	<0.001	0	NS
Cough	0.032	<0.001	0	NS
Pain in chest	0.017	<0.001	0	NS
Pain in other parts	0.009	<0.001	0	NS

2007; Gralla 2004). In the present study we confirm that a greater severity of disease symptoms affected the patients' quality of life. The most frequently diagnosed symptoms were chronic cough, dyspnea, chest pain, and hemoptysis. We also demonstrate that malnourished patients and those at risk of malnutrition had a lower quality of life in all functional domains and a greater severity of symptoms assessed with the EORTC QLQ C-30 and LC-13 questionnaires. These findings are in line with those of Gupta et al. (2006) who have shown that the score of well-nourished patients significantly better in quality of life concerning the global, physical, and role functions compared to malnourished patients. In that study, interestingly, the median role function score in well-nourished patients was 41.6 points higher than the corresponding score in malnourished patients, indicating a much better functioning from the patients' perspective. Likewise, quality of life scores on multiple symptom scales were significantly better among well-nourished patients.

The present study demonstrates that malnutrition significantly diminished quality of life in all functional scales, and enhanced the occurrence and intensity of cancer symptoms in the EORTC QLQ C-30 and LC-13 questionnaires. In multivariate analysis, malnutrition was a significant independent predictor of diminished quality of life in the physical functioning, and increased the severity of nausea, vomiting, and insomnia. These findings are consistent with those of other authors who demonstrate that a deterioration in quality of life is lower in physical, social, and cognitive domains is associated with body weight loss and with intensified clinical symptoms as shown in a study in 907 cancer patients (Nourissat et al. 2008). Concerning cancer-induced limitation in daily functioning and independence, a predicting power of abnormal weight loss is substantial. Ovesen et al. (1993) have shown that weight loss is related to limited performance and lower global health status in a group of lung, breast and ovarian cancer patients. Other authors also have demonstrated

that weight loss contributes to quality of life decrease and symptom severity increase, especially pain and fatigue, as assessed by the EORTC QLQ-C30 questionnaire (van Hazel et al. 1983).

In a study of Scott et al. (2003), nearly 40% of NSCLC patients displayed weight loss of over 5% and nearly 80% of them had an increased concentration of C-reactive protein (>10 mg/L). Patients whose weight loss exceeded 5% obtained lower Karnofsky performance and global quality of life scores, and experienced a greater severity of fatigue, and pain compared to patients with normal body weight. In other studies, weight loss has been an independent predictor of survival in patients after pulmonary resection (Scott et al. 2002).

Mohan et al. (2008) have shown that anorexia is one of the most frequent symptoms that occurs in up to 57% of stage III and IV lung cancer patients. Among patients who do not show a response to chemotherapy, there are persons who suffer from fever, anorexia and weight loss at the very beginning of treatment commencement. The authors believe that chemotherapy does not cause any significant improvement in respiratory function or nutritional status, and thus dos not improve quality of life. In the present study, as many as 86% of patients were treated with chemotherapy and over 30% underwent pulmonary resection.

5 Conclusions and Practical Implications

The findings of this study show that nutritional status could be used as an important stratification variable in studies evaluating quality of life outcomes in advanced lung cancer. A strong relation between quality of life and weight loss shows the importance of dietary management in patients with lung cancer. The management of NSCLC ought to take into account strategies to improve various aspects of quality of life and nutritional status to achieve comprehensive benefits. Nutritional care should be integrated into the global oncology care because of its significant contribution to quality of life.

Nutritional intervention should constitute a supportive element of any oncological treatment. Therapy should focus on symptom control, treatment tolerance improvement, and quality of life enhancement. The assessment of nutritional status ought to run parallel with that of quality of life to tailor nutritional interventions to individual patients' needs. As nutritional status might have a clinically significant influence on quality of life, nutritional support should constitute an integral element of any oncological treatment.

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

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 33: 27–36
DOI 10.1007/5584_2017_27
© Springer International Publishing Switzerland 2017
Published online: 21 July 2017

Influence of Vitamin D and Cotinine on T-Regulatory Cells and Asthma Severity in Children

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Abstract

Asthma is a common chronic respiratory diseases in children. Understanding the immune mechanisms of epigenetic factors may contribute to a better control of asthma. This study seeks to determine the effects of serum vitamin D and urine cotinine on asthma severity and on T regulatory cells (Tregs) and other immune-related factors such as CD3, CD4, CD8, CD19, CD16/56, and anti-CD3 HLA-DR3. The study involved 34 children with asthma. Disease severity was assessed with the Asthma Control Test, spirometry, and the fractional exhaled nitric oxide (FeNO). The control group consisted of 18 healthy children. We found a significantly lower proportion of Tregs in the serum of asthmatic children compared with the control group ($p < 0.002$). There were no significant differences in the other immunological factors investigated. Nor was there any appreciable association between vitamin D or cotinine and the course of asthma, FeNO, Tregs, and the other immune factors. However, the percentage of Tregs was positively associated with the level of FeNO ($p < 0.02$). In conclusion, the study shows a role of T regulatory cells in the pathogenesis of asthma in children, but fails to show any influence of serum vitamin D or urine cotinine on disease course.

Keywords

Asthma • Cotinine • Exhaled nitric oxide • Immune factors • Regulatory T-cells • Respiratory disease • Spirometry • Vitamin D

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1 Introduction

Bronchial asthma is a disease of complex etiology and clinical presentation. According to the guidelines of the National Asthma Education and Prevention Program (NAEPP 2007) asthma is a chronic disease of the respiratory system causing recurrent cough, shortness of breath, and wheezing. The symptoms result from airway obstruction and may resolve spontaneously or as a result of treatment (Boulet et al. 2015). Asthma is commonly included in a group of lifestyle diseases due to the interconnection between morbidity and the social development level, with morbidity rates higher in developed countries. The number of asthma sufferers in the world is estimated at around 300 m, 30 m of them in Europe alone. According to the WHO, the total number of asthmatics will have increased by another 100 m by 2025 (GINA Report 2014). Among children, asthma is the most common respiratory disease. According to the Epidemiology of Allergic Disorders in Poland study (ECAP 2016) conducted among children in two age groups: 6–7 and 13–14 yr old, the proportion of the asthma-affected is 24.5 and 17% for city dwellers and 16 and 11% for rural areas, respectively. Such results place Poland among the countries with a high incidence of allergy-related diseases.

Asthma pathogenesis is complex and, despite substantial research into its causes, there remains a lot to be learned. The cells responsible for chronic inflammation of airways belong mainly to mastocytes, neutrophils, T-lymphocytes, and macrophages. In asthma patients, Th2 lymphocytes (CD4+) are prevalent in airways, while in healthy individuals the Th1 cell response is the most common. Th2 lymphocytes produce cytokines taking a key part in maintaining inflammatory infiltration. The production of the interleukins IL-4 and IL-13 stimulates B lymphocytes to over-produce IgE; IL-5 is responsible for eosinophillia, and IL-9 leads to differentiation of mastocytes (Ingram and Kraft 2012; Klein et al. 2012; Barnes 2008). In normal conditions, the activity of this pathway may be inhibited by T-regulatory FoxP3 cells (Josefowicz et al. 2012). However, numerous studies indicate that both the number and function of the cells decline in allergic diseases (Lin et al. 2008; Lee et al. 2007).

There exist a number of external factors with a potential to influence the development and course of allergic diseases. Among lifestyle factors, vitamin D deficiency and exposure to tobacco smoke are of significance. Apart from its obvious role in calcium-phosphorus management (Ryan et al. 2013), vitamin D is an important hormone of the immune system. In the mid-1980s, Ohta et al. (1985) have shown in *in vitro* research that vitamin D stimulates proliferation and maturation of monocytes and macrophages. Its role in the immune system results from the presence of the nuclear vitamin D receptor in many immunocompetent cells. Research shows that vitamin D may inhibit the Th2-dependent inflammation of airways (Gorman et al. 2010). Vitamin D also increases the expression of anti-inflammatory cytokines such as IL-4, IL-5, and IL-10 and it facilitates the differentiation of CD4 lymphocytes into regulatory T-lymphocytes which, in turn, assist in controlling allergic reactions (Urry et al. 2012). Vitamin D deficiency is observed in the population with an increasing frequency; this results from the so called ‘Western lifestyle’ involving indoor occupations, low consumption of fish and other products rich in vitamin D, and a growing problem of obesity.

Detrimental effects of tobacco smoke on the human body, in particular on the respiratory system, are commonly known. Larsson et al. (2001) have shown an increased risk of bronchial asthma in adults exposed to tobacco smoke. According to the WHO, over 700 m children in the world may be passive tobacco smokers (Hassanzad et al. 2015). Such exposure to smoke may be particularly dangerous for children suffering from bronchial asthma as it amplifies the effect of other airway irritants, increases the incidence of exacerbation, and aggravates the disease course (Halterman et al. 2004). In controlling asthma it is therefore crucial, apart from the correct pharmacological

treatment, to motivate the parents to minimize the exposure of children to tobacco smoke.

A level of cotinine in the serum, urine, or saliva of a patient is a useful marker in the assessment of both active and passive smoking. Cotinine is a nicotine metabolite; its concentration enables to estimate the duration of passive exposure to tobacco smoke (Seccareccia et al. 2003). According to the research, even 30-min exposure to tobacco smoke causes an increase of cotinine concentration in the urine (Schick et al. 2013).

The aim of this study was to assess the effects of vitamin D and cotinine on regulatory T-lymphocytes and on the phenotype of peripheral blood (CD3, CD4, CD8, CD4/CD8, CD19, CD16/56, and CD3 anti-HLA–DR3) in children. An association between the concentrations of vitamin D and cotinine and the severity of asthma was also examined.

2 Methods

2.1 Patients

The research was conducted with the approval of the Bioethics Committee of the Military Institute of Medicine (permit no. 123/2014). Parents of the children qualified were informed of the research goals and signed the participation agreement. Thirty four children diagnosed with asthma took part in the study. The diagnosis was based on anamnesis and history of airway obstruction as recommended by the GINA (2015) guidelines. The selection criteria were age (minimum 6 yr old) and an increased level of IgE. Children exhibiting other accompanying respiratory system conditions, chest deformities, or unwilling to cooperate were excluded from the study. The control group consisted of 18 children with no allergy symptoms.

2.2 Level of Asthma Control

To assess the level of asthma control the Asthma Control Test was used (ACT). The ACT consists of five questions concerning the impact of asthma on daily activities, frequency of breathing difficulties, sleep disturbances, necessity of immediate use of bronchodilators, and a subjective evaluation of the level of asthma control by the patient. A score of 25 points signifies total control, 20–24 points adequate control, and the values below 19 points show the lack of illness control.

In addition, spirometry and nitric oxide content in the exhaled air (FeNO) measurements were conducted. Spirometry tests were conducted using a Lungtest 1,000 apparatus (MES, Cracow, Poland). The spirometry testing was evaluated according to the acceptability criteria of the American Thoracic Society (ATS)/European Respiratory Society (ERS) (Pellegrino et al. 2005). The following variables were recorded:

- FVC – forced vital capacity
- FEV1 – forced expiratory volume in the first second
- FEV1/FVC
- PEF – peak expiratory flow

The measurement of FeNO was conducted using a Medisoft apparatus (Sorinnes, Belgium). The tests were performed on an empty stomach.

2.3 Cotinine and Vitamin D Content

Cotinine concentration was measured in urine samples of 5 mL taken from all patients on the day of arrival to clinic. The measurement was done using an ELISA test (Abnova; Taipei, Taiwan) according to the manufacturer's recommendations. The detection limit was 1 ng/mL. Serum 25(OH)D concentration was measured with a chemiluminescent method using a Liaison analyzer (DiaSorin; Saluggia, Italy). The optimal concentration of vitamin D was considered as 30–50 ng/mL.

2.4 Lymphocytes Subpopulations

For the analysis pf lymphocyte phenotypes, peripheral blood samples (100 μL) were collected in the ethylenediaminetetraacetic acid (EDTA)-anticoagulated tubes. The samples were incubated for 20 min at room temperature with 20 μL of appropriate antibodies (BD Simultest™ – IMK Plus Kit; BD Biosciences, Warsaw Poland). Next, erythrocyte lysis (10 min, room temperature) was performed (BD FACS Lysing Solution; BD Biosciences, Warsaw, Poland). Afterwards the cells were washed twice with 2 mL of PBS and fixed in 200 μL of 1% paraformaldehyde in PBS. Then cells were counted in flow cytometry and analyzed by CellQuest Pro software (FACS Calibur, BD Biosciences, Warsaw, Poland). Additionally, percentage distributions of white blood cell subpopulations were assessed using CD45 and CD14 PE fluorescein-conjugated antibodies, and FSC (Forward Scatter) and SSC (Side Scatter) parameters, along the X-axis and Y-axis of flow cytometry determination, respectively. The results of lymphocyte phenotypes are presented as mean percentage of lymphocytes ± SD.

2.5 Natural T-Regulatory Cells (nTregs)

Whole blood samples (100 μL) were stained with 20 μL of primary antibodies CD4-PerCP, CD25-APC, CD127-FITC (BD Biosciences, Warsaw, Poland) or with appropriate isotype control for 20 min at room temperature. Next, erythrocyte lysis was performed for 10 min at room temperature (BD FACS Lysing Solution; BD Biosciences). The cells were washed twice with PBS and fixated and permeabilized in fixaton/permeabilization buffer in accordance with the manufacturer's recommendations (BD Pharmingen, BD Biosciences). After fixation, cells were stained with 20 μL of FoxP3 PE or isotype IgG1 kappa PE antibody (45 min/room temperature in the dark), then washed twice with PBS, fixed in 300 μL of 1% PFA in PBS solution and counted in a flow cytometer. Ten thousand counts of CD 4 PerCP positive cells stopped the acquisition. In the flow cytometry analysis (CellQuest™ Pro software; Becton Dickinson Polska) two gate restrictions were used: R1 – FSC/SSC lymphocytes and R2 – CD4 PerCP positive cells. The common parts of R1 and R2 (R1*R2 = R3) were used for further analysis. The results were presented as mean ± SD percentages of CD4+/CD25high, CD4+/CD25high/CD127low, and CD4+/CD25high/CD127low/FoxP3+ (nTregs) of population of cells located in R3 gate.

2.6 Statistical Analysis

Data were presented as means ± SD or medians with interquartile range (IQR) being equal to the difference between 25th and 75th percentile. Data distribution was checked with the Kołmogorow-Smirnov and Liliefors tests. Normally distributed group data were statistically compared with a paired or unpaired *t*-test, as required. Pearson's product-moment coefficient and Spearman's rank coefficient were used for the assessment of correlations between normal and non-normal distributed data, respectively. A p-value < 0.05 defined a statistically significant difference. Data analyses were performed using a commercial Statistica packet ver. 12 (StatSoft; Tulsa, OK).

3 Results

3.1 Immunoglobulin E, Vitamin D, and Cotinine Content

The median age of children in the asthma group was 8 yr (IQR, 6–12 yr), while in the control group it was 7 yr (IQR, 5–8 yr). The asthmatic children, on average, had a greater serum concentration of total IgE, 712 ± 40 vs. 44 ± 38 IU/mL in healthy controls ($p < 0.00001$), and a lower concentration of vitamin D, 23.0 ± 9.6 vs. 31.4 ± 9.6 ng/mL in healthy controls

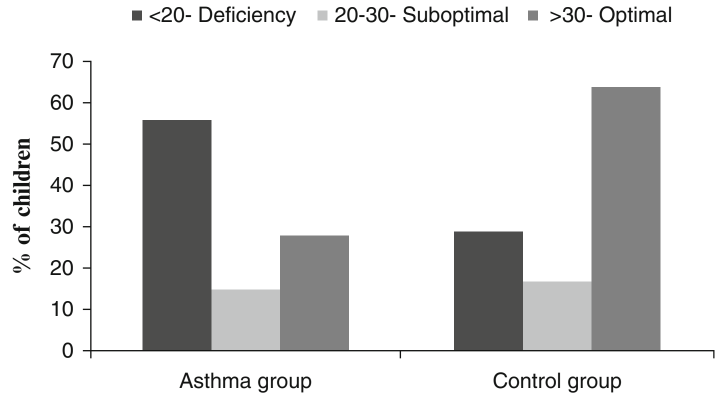

Fig. 1 Vitamin D content in asthmatic and healthy children

Table 1 Lung spirometry

FVC	93.6 ± 6.0
FEV1	91.3 ± 13.4
FEV1/FVC	88.3 ± 10.6
PEF	81.1 ± 19.8

Data are means ± SD of % predicted

($p < 0.04$). A large deficiency of vitamin D content (<20 ng/mL) was found in 56% of children with asthma. Figure 1 presents the range of vitamin D concentration in both groups of children. Thirty three percent of asthmatic children were exposed to tobacco smoke, which was reflected in increased urine cotinine content as opposed to none in the control group. Cotinine content was 3.9 ± 7.0 ng/mL in the tobacco smoke exposed vs. 0.1 ± 0.1 ng/mL in non-exposed children.

3.2 Lung Function

The average concentration of nitric oxide in exhaled air in asthmatic children was elevated to 22.4 ± 23.8 ppb. A good asthma control, as assayed using the ACT, were observed in 28% of the asthmatic children; 47% of parents estimated their children asthma control as insufficient. No obstructive symptoms were found in spirometry tests. The test results are shown in Table 1.

3.3 Lymphocyte Subpopulations

A proportion of regulatory T-lymphocytes was significantly lower in the asthmatic children compared to the control group ($p < 0.002$). There were no other appreciable differences in the remaining subpopulations of lymphocytes investigated between the asthma and control groups (Table 2).

3.4 Correlations

We found a significant correlation between the regulatory T-lymphocytes and exhaled NO content. The number of Tregs was in direct proportion to that of FeNO (Fig. 2). There were no other correlations apparent between the content of vitamin D, cotinine, and the proportion of natural regulatory T-lymphocytes and CD3, CD4, CD8, CD4/CD8, CD19, CD16/56, and CD3 anti-HLA-DR3. No influence of vitamin D and cotinine levels on asthma course were unravelled during the assessment with the Asthma Control Test, spirometry, or nitric oxide measurement in the exhaled air. Nor was vitamin D level found to affect the concentration of total IgE.

4 Discussion

This study demonstrates that vitamin D content is diminished in children with asthma compare with

Table 2 Percentages of cell subpopulations

	CD3	CD4	CD8	CD4/CD8 ratio	CD19	CD16/56	CD16/56/CD3	CD3 anti-HLADR	Tregs
Asthma	65.9 ± 7.5	34.4 ± 6.7	30.3 ± 4.8	1.2 ± 0.4	15.1 ± 4.6	13.7 ± 6.3	3.2 ± 1.7	6.8 ± 3.2	0.8 ± 0.6*
Control	62.1 ± 9.7	34.2 ± 7.6	27.1 ± 7.9	1.4 ± 0.5	21.3 ± 12.2	10.9 ± 5.6	2.3 ± 1.7	5.9 ± 4.0	2.1 ± 2.2

Data are mean ± D percent; $^*p < 0.002$

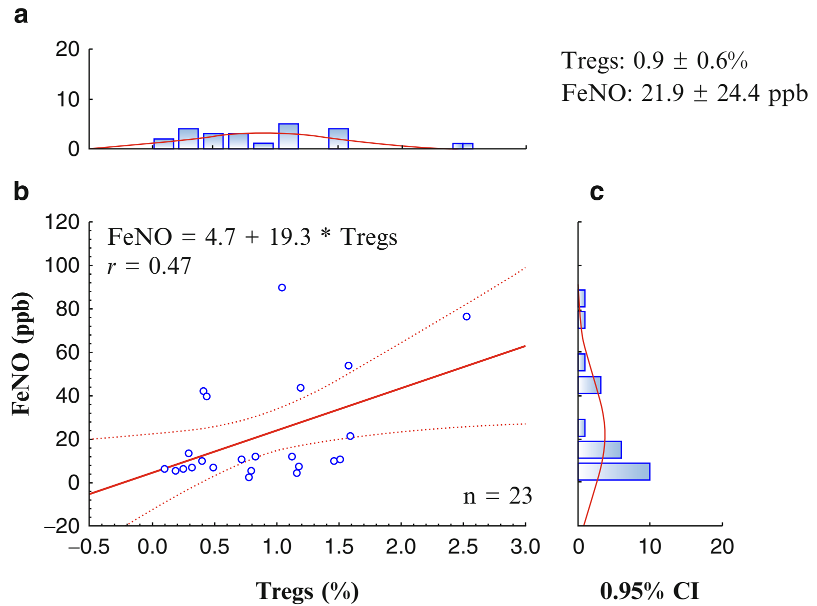

Fig. 2 Correlation between T regulatory cells (Tregs) and exhaled nitric oxide (FeNO)

their healthy peers. There are a number of studies that point to the role of vitamin D in the function of immune system and the development of allergy-related diseases, including bronchial asthma. Urry et al. (2012) have shown the influence of vitamin D on the production of IL-10 having anti-inflammatory properties in allergic diseases. Moreover, vitamin D causes activation of the CD4+ regulatory T-lymphocytes, including both Th1 and Th2 (Gregori et al. 2002; Meehan et al. 1992). Vitamin D, administered with dexamethasone, increases the therapeutic response to steroids in patients with steroid-resistant asthma (Xystrakis et al. 2006).

Chronic inflammatory infiltration in the respiratory tract is conducive to episodes of bronchial over-sensitivity and obstruction. Studies *in vitro* demonstrate that vitamin D may inhibit the development of airway smooth muscle cells induced by growth factor phosphorylation of retinoblastoma protein and checkpoint kinase (Damera et al. 2009). This molecular pathway has been confirmed *in vivo*, by showing the advantageous effects of vitamin D on spirometric indices of bronchial obstruction such as forced expiratory volume in 1 s (FEV1) and its fraction of forced vital capacity (FEV1/FVC) (Brehm et al. 2012).

There exist, however, studies presenting results divergent from those discussed above. Menon et al. (2012) have failed to demonstrate any interdependency between vitamin D content and the course of bronchial asthma; their research compared a group of nearly 300 asthmatic children with a similarly-sized control group. Likewise, Mai et al. (2012) have obtained analogous results in research on adults; nonetheless showing that a lower content of vitamin D is related to increased risk of asthma. Such results do not confirm the presence of a direct link between vitamin D content and asthma severity, although the possibility of greater risk of allergic diseases in case of a decreased content of vitamin D remains viable.

Another environmental factor that may influence the course of bronchial asthma is nicotine. As a result of exposure to tobacco smoke, epithelial cells of bronchial passages get damaged and an inflammatory process follows. Smoking attracts neutrophilis to, and increases their number in, the lungs (Schwartz and Weiss 1994; Hunninghake and Crystal 1983). In the present study we failed to demonstrate the association

between enhanced urine cotinine content and a proportion of regulatory FoxP3 T-lymphocytes or the phenotype of peripheral blood lymphocytes (CD3, CD4, CD8, CD4/CD8, CD19, CD16/56, and CD3 anti-HLA–DR3). Meng et al. (2012), however, have reported an increase in the proportion of regulatory FoxP3 T-lymphocytes in bronchoalveolar lavage fluid from rats exposed to tobacco smoke. Jiang et al. (2010) have reported a lower proportion of CD4 + CD25+ lymphocytes in rats immunized with ovalbumin (OVA+) and exposed to tobacco smoke compared with rats OVA(+) and OVA(−) that are not exposed to smoke. The immunized rats exposed to tobacco smoke also show a reduced expression FoxP3 protein.

The literature demonstrates discrepancies concerning the effect of regulatory lymphocytes on asthma severity. Some authors report no increase in CD4(+)CD25(+) lymphocytes during the escalation of asthma symptoms (Shi and Qin 2005). Others demonstrate a drop in natural regulatory T-lymphocytes in asthma flare-ups (Yang et al. 2013; Xue et al. 2007). There is limited research assessing the relationship between the proportion of regulatory T-lymphocytes and spirometry or FeNO results. The present study demonstrates an association between FeNO and Tregs in asthmatic children. A similar attempt to correlate FeNO and Tregs has been undertaken by Eszes et al. (2012) in a study that involved 22 pregnant women diagnosed with asthma. Those authors have failed to substantiate the presence of any appreciable correlation between regulatory T-lymphocytes and FeNO. Ismail et al. (2015) have suggested that serum vitamin D content and/or the proportion of regulatory T-lymphocytes may be useful indices in predicting the asthma severity. The authors report that the serum 1,25(OH)D3 content and the proportion of FoxP3 T-lymphocytes is decreased in asthmatic children. They also show a larger drop in Tregs in children with severe asthma compared with moderate or mild asthma cases; the differences were not, however, statistically significant. Similar findings have been reported by Yang et al. (2013) who show an inverse association between the number of Tregs and asthma severity in a group of 150 asthmatic children.

In conclusion, the findings of the present study demonstrate that regulatory T-lymphocytes have a regulatory role in the pathogenesis of asthma in children as judged from the association between Tregs and FeNO. However, we failed to demonstrate any influence of serum vitamin D or urine cotinine content on asthma course, and any interrelations between vitamin D or cotinine and regulatory T-lymphocytes.

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

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 33: 37–42
DOI 10.1007/5584_2017_26
© Springer International Publishing Switzerland 2017
Published online: 17 June 2017

Identification of *Mycobacterium* Species by MALDI-TOF Mass Spectrometry

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Abstract

Matrix-assisted laser desorption ionization – time-of-flight (MALDI-TOF) mass spectrometry enables to identify microorganisms by comparison of the protein content with reference spectra in the database. The aim of this study was to evaluate the efficacy of phenotypic identification of mycobacteria by MALDI-TOF mass spectrometry in laboratory practice. Seventy five isolates of mycobacteria were identified by molecular and phenotypic method, and the results were compared by MALDI-TOF. For MALDI-TOF, material was processed according to the Bruker Daltonics protocol and Mycobacterial Library database version 2.0, with 313 reference mycobacteria spectra. All except one of the 72 isolates agreed with regard to the species and genus by both methods. Forty three isolates were identified as the *M. tuberculosis* complex by MALDI-TOF. Thirty one isolates of nontuberculous mycobacteria were consistently identified by both methods to the species level. We conclude that MALDI-TOF mass spectrometry is an accurate method of bacterial identification. Simplicity, speed, and economic availability of the method makes it suitable for mycobacteria identification in a routine laboratory.

Keywords

Bacterial isolates • Mass spectrometry • *Mycobacterium tuberculosis* • Phenotype bacterial identification • Protein identification

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1 Introduction

Mycobacterial infections are a public health problem causing significant morbidity and mortality (Zumla et al. 2013; Jarzembowski and Young 2008). Tuberculosis (TB) is contagious, airborne, and a top infectious killer worldwide, although it is a curable and preventable disease. A WHO report indicates that 9.6 million people (5.4 million men, 3.2 million women, and 1.0 million children) fell ill with *M. tuberculosis* and 1.5 million people died from tuberculosis in 2014. Rapid and accurate diagnosis of mycobacterial infections is important for initiating early treatment and for preventing drug resistance (Balada-Llasat et al. 2013).

It is essential to differentiate mycobacterial isolates to the species level to detect if they are true pathogens or environmental contaminants. Tuberculous mycobacteria belonging to the *Mycobacterium tuberculosis* complex are of notable clinical importance. Likewise, a large group of nontuberculous mycobacteria are of clinical interest, such slowly growing *M. avium-M. intracellulare* complex, *M. kansasii, M. marinum, M. xenopi, M. simiae,* and *M. ulcerans*, and rapidly growing *M. abscessus, M. chelonae*, and *M. fortuitum* (Balada-Llasat et al. 2013). Conventional methods for the identification of mycobacterial species are simple to perform, but they require extensive incubation period, as long as 12 weeks. Several new tests have become available for detection of *Mycobacterium* species. Molecular methods are rapid, but expensive and often exclusive to reference laboratories. Matrix-assisted laser desorption ionization – time-of-flight (MALDI-TOF) technology, used in mass spectrometry for the analysis of biomolecules, presents an alternative method for the identification and differentiation of mycobacteria. The method is increasingly on the rise as early and rapid identification of mycobacteria is essential for disease control (Dixon et al. 2015; Quinlan et al. 2015; Biswas and Rolain 2013; Tonolla et al. 2010). The increase in the use of MALDI-TOF is reflected in the outstanding rise in the number of publications on the subject, from one in 1995 to 395 in 2015.

The present study demonstrates the efficacy of MALDI-TOF mass spectrometry for the phenotypic identification of mycobacteria isolates from clinical material as compared with conventional methods. We also discuss the use of MALDI-TOF mass spectrometry for the diagnosis of fastidious bacteria, and the advantages and limitations of MALDI-TOF compared with other currently used identification methods in clinical laboratory.

2 Methods

The study was approved by the Ethics Committee of Jessenius Faculty of Medicine in Martin, Slovakia.

2.1 MALDI-TOF Mass Spectrometry

MALDI-TOF analysis identifies bacteria on the basis of their protein profile. The identification is based on comparison of the mass spectra of bacterial proteins with the known protein reference spectra in the database. Samples of microbial material are mixed with a matrix on a conductive metal plate, which results in the crystallization of a sample within the matrix. Then, the metal plate is introduced in the mass spectrometer where it is bombarded with brief pulses of nitrogen laser. The matrix absorbs laser energy, which leads to desorption of bioanalytes that are subsequently vaporized and ionized in the gas phase. The ionized molecules are accelerated in the electrostatic field and are ejected through a metal flight tube that is subjected to a vacuum until they reach a detector, with smaller ions traveling faster than larger ions. Thus, analytes are separated according to their TOF, which creates a mass spectrum that is composed by mass to charge ratio (m/z) peaks with varying intensities. The spectrum is a microbial fingerprint that is compared with a database for the identification at the species or genus level (Croxatto et al. 2012). MALDI-TOF MS is a precise, fast, and relatively

Table 1 Identification of mycobacteria by conventional/molecular and MALDI-TOF MS methods

Isolates (n)	Conventional/molecular	MALDI-TOF MS
43	*Mycobacterium tuberculosis*	*Mycobacterium tuberculosis* complex
4	*Mycobacterium avium*	*Mycobacterium avium*
2	*Mycobacterium fortuitum*	*Mycobacterium fortuitum*
1	*Mycobacterium chelonae*	*Mycobacterium chelonae*
3	*Mycobacterium kansasii*	*Mycobacterium kansasii*
1	*Mycobacterium* spp.	*Mycobacterium novocastrense*
7	*Mycobacterium xenopi*	*Mycobacterium xenopi*
8	*Mycobacterium gordonae*	*Mycobacterium gordonae*
6	*Mycobacterium intracellulare*	*Mycobacterium chimaera-intracellulare* complex

inexpensive method and a number of studies have confirmed its suitability in laboratory practice (Quinlan et al. 2015; Biswas and Rolain 2013; Balada-Llasat et al. 2013; Clark et al. 2013).

2.2 Study Design

Seventy five isolates of mycobacteria were identified by molecular and phenotypic identification methods, and they were compared with the identification by MALDI-TOF MS. Most of the isolates (72 isolates) came from clinical material (clinical strains previously isolated from patient specimens). There were only three isolates from an external control laboratory.

2.3 Identification of Mycobacteria by Conventional Methods

Mycobacteria were cultured on solid Ogawa's medium and liquid Sula's medium. They were identified by conventional methods: microscopy of Ziehl-Neelsen stained preparations, growth rate, and ability to growth at different temperatures, shape and pigmentation of the colonies, growth on the thiophene-2-carboxylic acid hydrazide (TCH) medium, nitrate-niacin test, and sensitivity to antibiotics.

2.4 Identification of Mycobacteria by MALDI-TOF Mass Spectrometry

For MALDI-TOF MS identification, material was processed according to the optimized extraction protocol for mycobacteria of Bruker Daltonics GmbH (Bremen, Germany). The protocol used zirconia-silica beads and the database of Mycobacteria Library (v2.0 Bruker Daltonics) with 313 reference spectra of mycobacteria. The obtained spectra from clinical samples were analyzed against spectra in the database using the Biotyper software. The reliability of identification obtained by MALDI-TOF MS was assessed from the best match based on the m/z ratio and from the logarithmic score ranging from 0 to 3. The scores ≥ 2.000 were interpreted for genus- and species-level identification, scores between 1.700 and 1.999 for genus-level identification, and the scores <1.700 were assumed unreliable for identification.

3 Results

All but one out of the 75 isolates were determined in conformity to the genus, species, or complex with both conventional and MALDI-TOF MS methods. The results are shown in Table 1.

The results obtained with MALDI-TOF MS corresponded to those obtained with conventional methods in case of 71 out of the 72 isolates from clinical material. Forty three isolates of *M. tuberculosis* were identified by MALDI-

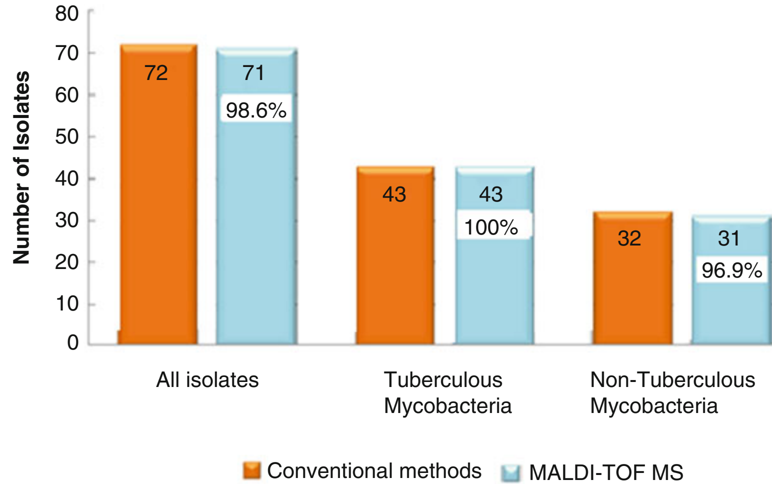

Fig. 1 Identification of tuberculous and non-tuberculous mycobacteria by conventional and MALDI-TOF MS methods

TOF MS as the *M. tuberculosis* complex, with 100% success. Thirty one out of the 32 isolates of non-tuberculous mycobacteria were consistently identified with both methods to the species or complex level (Fig. 1). The closely related species, identified consistently within a complex, were considered identical identification. One isolate, identified as *M. novocastrense* by MALDI-TOF MS, was designated as *Mycobacterium* species with hybridization techniques. All non-tuberculous mycobacterium strains were confirmed by PCR in the reference laboratory of the National Institute for Tuberculosis, Lung Diseases and Thoracic Surgery in Vysne Hagy, Slovakia.

4 Discussion

Mycobacteria cause significant morbidity in humans. Rapid and accurate identification of mycobacteria is important for the improvement of patient outcomes. Several diagnostic techniques, such as biochemical, sequencing, and probe methods, are used for mycobacterial identification (Balada-Llasat et al. 2013). Unambiguous diagnosis of active tuberculosis is a time-consuming process, requiring as long as 12 weeks for positive identification of the organism. This long time frame presents challenges for case identification. Early identification of mycobacteria is essential for the disease control (Biswas and Rolain 2013). MALDI-TOF MS is a powerful method for the detection and identification of proteins by molecular weight determination of individual, specific fragments. The recent developments of MALDI-TOF MS are rapidly changing the routine diagnostics scene. The method is accurate and easy to use, allowing for a quick determination of molecular protein weight, with minimal sample requirements (Benagli et al. 2011).

In the present study, MALDI-TOF MS correctly identified 98.6% of mycobacterial isolates from clinical material to the genus, species, or complex level. Tuberculous mycobacteria were identified as the *M. tuberculosis* complex with 100% success. For non-tuberculous mycobacteria, the results of MALDI-TOF MS corresponded to the conventional methods with 96.9% success. Only was one isolate imprecisely identified. It was designated as *M. novocastrense* by MALDI-TOF MS and as *Mycobacterium* species with hybridization techniques. *M. novocastrense* is rapidly growing photochromogenic mycobacterium, with yellow pigmented colonies when incubated in the light, which was confirmed by phenotypic identification. The identification of *M. novocastrense* was confirmed by PCR.

MALDI-TOF MS has many advantages. The method is quick, reliable, and cost-effective compared to conventional and molecular techniques. There are available extraction protocols for mycobacteria. The identification of mycobacteria is possible within 1–2 h. Databases of protein spectra, used as reference for comparison, are continually expanded by adding new spectra of mycobacteria since the identification of microorganisms is limited by the database (Benagli et al. 2011). The MALDI-TOF MS method has some other limitations concerning its use for the identification of mycobacteria due mainly to the high pathogenicity of some of these microorganism and the structure of their cell wall, requiring inactivation and special protein extraction protocols (Alcaide et al. 2016).

The genus Mycobacterium includes, as major groups, the pathogens of the *M. tuberculosis* complex and the non-tuberculous mycobacteria. The members of non-tuberculous mycobacteria are cited increasingly as the cause for opportunistic infections among immune-compromised patients. This trend and the rise of antibiotic resistance in this genus necessitate improved differentiation of mycobacteria. Although MALDI-TOF MS accurately classifies isolates as members of *M. tuberculosis* complex, it is unable to separate them into individual species. While MALDI-TOF MS can differentiate between *M. chelonae* and the *M. abscessus* group, and between *M. avium* and *M. intracellulare*, it cannot differentiate *M. intracellulare* from *M. chimera, and M. massiliense* from *M. abscessus* (Saleeb et al. 2011). The differentiation of *M. massiliense* from *M. abscessus* complex is of clinical interest because *M. massiliense* is one of the subspecies of *M. abscessus* complex and exhibits higher rates of response to antibiotic treatment for lung infection than do the other members of that complex (Kehrmann et al. 2016). The currently available databases of reference spectra of known proteins need extension, especially for less common organisms. New MALDI Biotyper System v3.0, created in 2015, contains 542 additional references of mainly clinical isolates. This new database includes a total of 855 reference spectra from 149 species (Pranada et al. 2015). A new Mycobacteria Library v4.0 covers 159 of the currently known 169 *Mycobacterium* species. Eight hundred and eighty strains, of which more than 450 are clinical isolates, cover the natural variability of *Mycobacterium* species. This latest version secures high sensitivity of mycobacteria identifications.

In the laboratory diagnosis of tuberculosis, smear microscopy is usually confirmed by culture. That is the gold standard which, however, requires approximately 45 days of incubation time. Automated and semi-automated liquid culture systems have reduced the culture time and increased sensitivity. Yet conventional methods used for the identification of tuberculosis still have low sensitivity (Şamlı and İlki 2016). The identification of tuberculous and non-tuberculous mycobacteria, based on conventional phenotypic tests, is time-consuming, labor-intensive, expensive, and often provides erroneous or inconclusive results (Griffith et al. 2007). Molecular methodologies, although rapid, are expensive, need different genetic markers, and are often exclusive to reference laboratories (Quinlan et al. 2015). For the identification of bacteria, which are difficult to culture, MALDI-TOF MS is a powerful, rapid, precise, and cost-effective method, compared to conventional phenotypic or molecular techniques (Biswas and Rolain 2013).

The cost of MALDI-TOF MS identification is significantly less compared to other methods, including genomic sequencing or biochemical techniques. MALDI-TOF MS generates less waste than other methods that are based on molecular and biochemical tests that use many disposable materials (Balada-Llasat et al. 2013). The identification of mycobacteria from isolates with the use of MALDI-TOF MS takes approximately 1–2 h, compared to a few weeks of other phenotypic identification, which has brought into laboratory practice a revolutionary shift in the speed and accuracy of identification.

5 Conclusions

MALDI-TOF MS is a diagnostic method which clarifies and accelerates the diagnosis, especially in slow growing bacteria, which are difficult to culture, such as *Mycobacterium* species. This method is a new laboratory option for the diagnosis of clinical infections and a valid alternative to conventional methods. In this study we confirmed that MALDI-TOF MS represents a rapid, reliable, and cost-effective identification technique and the method of choice for the identification of clinically important *Mycobacterium* species in a routine laboratory. Rapid and accurate diagnosis of mycobacterial infections is essential for the commencing of early treatment and the prevention of disease spread from person to person.

Acknowledgments Supported by project KEGA 032UK-4/2015 – Modern Education for Knowledge Society of the Ministry of Education, Slovak Republic.

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

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 33: 43–53
DOI 10.1007/5584_2017_31
© Springer International Publishing Switzerland 2017
Published online: 2 June 2017

Indoor Exposure to Volatile Organic Compounds in Children: Health Risk Assessment in the Context of Physiological Development

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Abstract

Indoor air quality is strongly affected by the contamination of ambient air and that related to building and finishing materials and to human activity. Poor ventilation of closed spaces facilitates retention of greater quantity of pollutants. Infants and children are at particular risk of exposure to indoor air pollutants as they undergo rapid physiological and biochemical changes and demonstrate activity patterns unlike those in adults. Health risk assessment in children should be carried out with regard to children-specific factors, since these factors may constitute a source of errors. In this article we weigh up two different: Scenario 1 in which risk assessment was carried out in five age-groups (0–1, 2–3, 4–6, 7–11, and 12–16 years of age) and Scenario 2 encompassing only two age-groups (0–6 and 7–16 years of age). The findings indicate that data on carcinogenic and non-carcinogenic effects obtained by applying the second scenario were overestimated or averaged; either giving much reduced information that may lead to a false judgment on actual risk. This kind of fallacy is avoided when applying the age stratification into a greater number of groups for the health risk assessment in children.

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Keywords
Children • Exposure • Health risk assessment • Indoor air • Volatile organic pollutants

1 Introduction

In the twenty-first century a great attention has been put to the environment, as a factor strongly contributing to human health (Lin et al. 2016; Saenen et al. 2016; Suk et al. 2016; Webster et al. 2016; Lagidze et al. 2015; Hänninen et al. 2011; Luo et al. 2011; Remy et al. 2011; Qian et al. 2007; Sherriff et al. 2005; Diez et al. 2000). Since humans spend up to 90% of the time indoors, this area of research is a subject of special interest. Indoor air quality is strongly affected by contaminants related to building, finishing materials (paints, wallpapers, glues, and varnishes and solvents), and daily human activities (tobacco smoke, cleaning agents, and cosmetics) (Billionnet et al. 2011; Etzel 2007). Poor ventilation of a closed space raises the issue of retention of pollutants and thus magnification of their quantities. Thus, exposure to pollutants *via* the respiratory tract is a frequent subject of public concern. The largest group of air contaminants constitute volatile organic compounds (VOCs) that cause a variety of detrimental health effects (Table 1).

Children are of distinct concern when it comes to the exposure of indoor air to pollutants. Children can be exposed to VOCs not only at home but also in a variety of different places such as nurseries, kindergartens, and schools. What distinguishes children from adults is their activity patterns, and biochemical and physiological parameters. Due to an incomplete physiological development, a child's ability to metabolize certain chemical agents is strongly reduced (Cohen Hubal et al. 2014; Ginsberg et al. 2004). The greatest susceptibility to chemical compounds can be observed in newborns and infants (Scheuplein et al. 2002). Children have a much higher lung area *per* body mass ratio than adults do. On average, a one-year old child inhales as much as 7 mL/min/kg of air, whereas an adult does 3–5 mL/min/kg (US EPA 1997). This suggests that while inhaling a relatively smaller amount of air pollutants than adults, the amount of air passing through the child's lungs is two times larger in volume per kilogram of body mass. At the age of two years, child's biochemical and physiological characteristics are almost fully developed. However, differences between children and adults do not disappear until the maturity time. The maturation of reproductive, endocrine systems, and nervous systems is a slow process that remains most susceptible to disruptive effects of pollutants (Selevan et al. 2000). For this reason, the assessment of health risk of

Table 1 Basic characteristics of common volatile organic compounds (VOCs)

Compound	Source	Health effects
Formaldehyde	Building and finishing materials, varnishes, glues, insulating materials, furniture, tobacco smoke	Irritation of mucous membranes, respiratory inflammation, headaches, carcinogenesis
Phenol	Plasticizers, glues, insulating materials, roofing paper	Headaches, dizziness, nausea, disruptive effect on liver and kidneys, carcinogenesis
Toluene	Varnishes, paints, cleaning agents, glues, insulating materials, glues	Disruptive effect on nervous system, skin irritation, liver impairments
Xylene (o-, m-, p-)	Varnishes, paints, cleaning agents, glues, impregnates, furniture	Brain depressive effects, mucous membranes irritation, respiratory inflammation
Benzene	Varnishes, paints, cleaning agents, glues, synthetic materials	Headaches, dizziness, nausea, disruptive effect on liver and kidneys, carcinogenesis, anemia, leukemia

children exposed to a vast variety of environmental pollutants should be carried out with caution. This principle applies in particular to newborns and infants as their physiological parameters undergo large changes over a short period of time. The pace of changes is often a source of methodological mistakes while applying standard risk assessment procedure (FAO and WHO 2009; WHO 2010; Kyle et al. 2006). Such mistakes consists of omitting children while carrying out the procedure, treating them as belonging to the entire uniform population (Guo et al. 2004; Wcisło et al. 2002), or distinguishing too few age-groups (Hoddinott and Lee 2000). In each instance, risk assessment would be incomplete or burdened with uncertainty, which is due to data averaging of exposure patterns and physiological changes rather than treating them in the age-specific way. Thus, evaluation of health effects resulting from a given exposure would not be properly fulfilled. Furthermore, health assessment, which is expected to be a source of information enabling to manage risks in an economic and environmental-responsible manner, may eventually contribute to a misplacement of resources and an improper protection of individuals.

While carrying out the risk assessment, main attention is usually directed toward the identification of the actual exposure time and magnitude, whereas relatively less toward to the methodology itself. Since risk parameters are mathematically related, a mistake made once recurs along the entire procedure, compounding the error. Therefore, the aim of this article was to draw attention to the introduction of health risk assessment in sequential age-groups of children, rather than in age-unlimited across-the-board routine, concerning the indoor exposure to VOCs. We also elucidated the issue of an excessive methodological simplification and emphasized the importance of methodological consistency in health risk assessment in children to ensure a better protection against toxic chemical compounds.

2 Methods

2.1 Chemical Pollutants

This retrospective study used databases; therefore the requirement for ethical permission was waived by the Ethics Committee. Data presented in this study were gathered by the Department of Environmental Protection of the Research Institute of Building Technique in Warsaw, Poland in the years 1986–1997 and served as the basis for HIGMAT Central Data Base B. The indoor air quality was analyzed in residential and public buildings across Poland. The results demonstrate the presence of over 100 substances. 40 of them were identified as having a negative effect on human health. The greatest impact on sanitary and health conditions had 12 substances. Those were the following: formaldehyde, phenol, toluene, xylenes (o-, m-, p-), naphthalene, benzene, butyl acetate, 1-chloronaphthalene, butyl alcohol, chlorophenols, 1- and 2-chlorophenol, and ethyl acetate. The indoor tests of air quality were carried out in 34 nurseries, 496 kindergartens, and 412 schools.

2.2 Study Protocol

The methodology of risk assessment corresponded with that used by The United States Environmental Protection Agency (US EPA 2000) and consisted of risk identification and characterization, and exposure and toxicity assessments (Guo et al. 2004). Two age-related scenarios were considered. The first scenario consisted of risk analysis in children divided into five sequential age-groups of 0–1, 2–3, 4–6, 7–11, and 12–16 years. The rational for multiple groups stemmed from age-related differences in children’s activity and the place of its performance as suggested by other studies (Cohen Hubal et al. 2014; Stroop et al. 2002). The exposure data demonstrate that each place investigated had a significantly different

Table 2 Indoor air concentration; average and maximum

Compound	Nursery		Kindergarten		School	
	Avg. (μg/m^3)	Max. (μg/m^3)	Avg. (μg/m^3)	Max. (μg/m^3)	Avg. (μg/m^3)	Max. (μg/m^3)
Formaldehyde	32	40	96	128	55	73
Phenol	8	11	14	23	12	19
Toluene	130	166	90	140	129	209
Xylenes (o-, m-, p-)	596	660	97	171	202	209
Naphthalene	na	na	3	6	8	11
Benzene	16	26	79	131	302	322
Butyl acetate	882	965	86	121	174	231
1-chloronaphthalene	na	na	na	na	5	6
Butyl alcohol	93	131	78	97	229	329
Chlorophenols	na	na	5	6	17	19
1- and 2-chlorophenols	na	na	3	3	na	na
Ethyl acetate	17	31	91	116	194	306

na non-available data

concentration of the 12 pollutants of interest. Additionally, nursery and school groups were stratified into two subgroups with regard to physiological differences in activity patterns. The second scenario, for comparison, consisted of just two groups of children of 0–6 and 7–16 years of age.

2.3 Assessment of Health Effects of Pollutants

The objective was to assess the health risk connected with the presence of VOCs. Those compounds mainly originate from building and finishing materials, furniture, or cleaning agents (Table 1). The compounds of utmost concern are the following: formaldehyde, phenol, toluene, xylene, naphthalene, butyl acetate, butyl alcohol, ethyl acetate and chlorinated aromatic, and poliaromatic polluting agents. The analytical data concerning the presence of VOCs at the sampling sites are presented in Table 2. The assessment of health effects connected with the exposure imposes difficulties due to diverse sources of VOCs emissions, limited knowledge on additive effects of various compounds, and usually a long time exposure at relatively low concentrations.

2.4 Assessment of Age-Related Exposure Scenarios

The assessment of exposure risk includes the estimation of basic human parameters connected with VOCs such as inhalation rates, exposure duration, body weight, and daily activity pattern. All these data, specific for the inhalation route of exposure, were taken from the US EPA Exposure Factors Handbook (US EPA 1997).

Scenario 1 (Detailed Age-Related Analysis)
For this scenario we assumed that a child spends in nursery 45 h *per* week, 40 h in kindergarten, and 33 h at school (exposure duration). The exposure frequency was taken as 44, 40, and 40 weeks *per* year for nurseries, kindergartens, and schools, respectively. The assumed number of years spent in each place was: 2.5 year in nursery (from age 6 months until 3 years), 3 years in kindergarten, and 10 years at school (up to age 16). The inhalation rates, daily activity patterns, and averaged body weights were obtained from US EPA (1997) (see in Tables A, B, and C Supplementary material). Multiplying the inhalation rate specific for each activity by the percentage of daily activity pattern an average inhalation rate specific for each type of activity was obtained (see equation below). The sum of activities characteristic for each age-group of

Table 3 Cancer slope factor (CSF) and reference concentration (RfC) for volatile organic compunds (VOCs) present in nurseries, kindergartens, and schools

Compound	CSF $(\mu g/m^3)^{-1}$	RfC (mg/m^3)
Formaldehyde	1.3E-05	0.0098
Phenol	–	0.2
Toluene	–	5
Xylenes (o-. m-. and p-)	–	0.1
Naphthalene	–	0.003
Benzene	7.8E-06	0.03
Butyl acetate[a]	–	–
1-chloronaphthalene[a]	–	–
Butyl alcohol	–	30
Chlorophenols[a]	–	–
1- and 2-chlorophenols[a]	–	–
Ethyl acetate[a]	–	–

[a]Due to the missing data on CSF and RfC. the compound was excluded from risk calculations

children constitutes a total amount of air inhaled during a 24-h period of time.

$$IR_{tw} = IR_{sl} * \%t_{sl} + IR_{pass} \%t_{pass} + IR_{li} * \%t_{li} + IR_{mi} * \%t_{mi} + IR_{hi} * \%t_{hi}$$

where: IR_{tw} is time weighted inhalation rate; IR_{sl}, IR_{pass}, IR_{li}, IR_{mi}, IR_{hi} are inhalation rates specific for sleep, passive state, light intensity, moderate intensity, and high intensity, respectively; and $\%t_{sl}$, $\%t_{pass}$, $\%t_{li}$, $\%t_{mi}$, and $\%t_{hi}$ are the percentage times of sleep, passive state, light intensity, moderate intensity, and high intensity, respectively.

Scenario 2 (Simplified Age-Related Analysis) This scenario required averaging the data assumed for Scenario 1. Children 6 months to 6 years of age spend in nursery and kindergarten, on average, 43 h *per* week and 43 weeks *per* year, while children 7–16 years of age spend as much as 33 h *per* week and 40 weeks *per* year at school.

Either scenario took under consideration also the highest exposure situation. Exposures to VOCs were separately calculated for non-carcinogenic and carcinogenic effects. For the non-carcinogenic effect, the averaged time of exposure amounted to 2.5 years in nursery, 3 years in kindergarten, and 10 years at school (Scenario 1) and 6.5 years in nursery and kindergarten. and 10 years at school (Scenario 2). The number of days over which the exposure took place was 220 days in nurseries, and 200 in kindergartens and schools (scenario 1), while in scenario 2 it amounted to 213 and 200 days for children 6 months to 6 years of age and 7–16 years of age, respectively. For the carcinogenic effect, the averaged time of exposure was 70 years and the number of days over which the exposure took place was 365 days.

2.5 Toxicity Assessment and Risk Characterization

Toxicity assessment for indoor VOCs in nursery, kindergarten, and school was based on the available scientific data on adverse effects that might be caused in exposed children and youngsters. This assessment included the identification of toxicity measures for each VOC present in US EPA (2002), data of reference concentration (RfC) for the evaluation of non-carcinogenic effects, and cancer slope factor (CSF) for the evaluation of carcinogenic effects (Davis et al. 2011) (Table 3).

The risk assessment procedure gathers data from the previous elaborative steps. The summary is in the form of numerically expressed risk. It was subdivided into two parts separately considering non-carcinogenic and carcinogenic health effects.

2.5.1 Non-cancer Risk

The presence of VOCs, especially formaldehyde, in the indoor air triggers the development of asthma in children (Rumchev et al. 2004; Rumchev et al. 2002). Formaldehyde is also responsible for atopy (Garrett et al. 1999), raised levels of specific immunoglobulin E (Wantke et al. 1996), and the inflammation of the respiratory tract in children (Franklin et al. 2000). Other symptoms of the indoor air pollution are the following: influenza, fatigue, headache, dizziness, nausea and vomiting, cognitive impairment, and tachycardia.

Non-cancer risk is expressed by the hazard quotient (HQ). The HQ assumes that a substance with non-carcinogenic effects exhibits a threshold response. In other words, no adverse effects would be noticeable below a certain exposure level. HQ value is unitless and is calculated according to formula:

$$HQ = CDI/RfC$$

where CDI is chronic daily intake (mg/kg/day) and RfC is reference concentration (mg/kg/day).

The CDI value is calculated by applying the following equation:

$$CDI = (VOC \times IR \times T \times F \times D) / (BW \times ATL \times NY \times 1000)$$

where VOC is the concentration of a given volatile organic pollutant (μg/m^3), IR is inhalation rate (m^3/h), T is the length of exposure (years), D is exposure duration (h/week), F is exposure frequency (weeks/year), BW is body weight (kg), ATL is averaged time of exposure (70 years for carcinogens) (years), and NY is the number of days over which the exposure takes place during the one year period (US EPA 1989).

In the situation when a population is exposed to more than one pollutant, additive effects are assumed. Thus, total non-carcinogenic effect is calculated as the sum of HQ values of each chemical and expressed as the hazard index (HI):

$$HI = HQ_1 + HQ_2 + \ldots + HQ_n$$

The United States Environmental Protection Agency's (EPA) 'Superfund' Program to clean up the nation's uncontrolled hazardous waste sites has developed an approach that there exists a certain probability for non-cancirogenic effects if either HQ (in case of single substance exposure) or HI (multi-substance exposure) is higher than unity (US EPA 1989).

2.5.2 Cancer Risk

Although the incident of childhood cancer remains scarce, there has been increased concern about the link between environmental factors, including exposure to toxic substances, and childhood cancer during the past two decades. In the US alone, childhood cancer is one of the frequent causes of death in children aged 0–14. More than 8000 such cases are there diagnosed each year (Gouveia-Vigeant and Tickner 2003). The same trend persists in Europe where an age-standardized tumor incidence rate for children aged 0–14 is 138.5 *per* 1 million children (Kaatsch 2010; Gatta et al. 2005; Coebergh et al. 2001). Cancer development is often attributed to genetic predisposition, but environmental exposure to toxic compounds may also play a substantial role. Naturally, only a small fraction of children exposed to cancer-inducing factors goes on to develop cancer at an early stage of life. Nevertheless, exposures during intrauterine life or in early childhood may result in the development of cancer during later childhood or adult life (Landrigan et al. 2004). In 2000, the US EPA composed a list of compounds strongly contributing to indoor air pollution. Those compounds (mainly VOCs) have been related to mucous membrane irritations, migraines, physiological damage, and cancer (Rumchev et al. 2004; Diez et al. 2000). Of the compounds in question, formaldehyde predominates, being present in almost all closed compartments and is a proven carcinogen (IARC 1999; Waters et al. 1999; Albert 1989). Studies on the carcinogenic effects of benzene are very limited and a possible connection between developmental exposure to benzene and cancer in children remains equivocal (Brosselin et al. 2009; Weng et al. 2009; Gunier et al. 2008; Whitworth et al. 2008; Knox 2005; Crosignani et al. 2004). However, scarce evidence suggests that benzene may have similar

cancirogenic effects in children as it has in adults (US EPA 2002; Reynolds et al. 2003).

The assessment of cancer risk represents the incremental probability that an individual will develop cancer over a lifetime as a result of specific exposure to a carcinogenic chemical according to the following formula (US EPA 1989):

$$CR = CDI \times CSF$$

where CR is cancer risk, CDI is chronic daily intake (mg/kg/day), and CSF is cancer slope factor $(mg/kg/day)^{-1}$.

If exposure takes place to a mixture of chemicals, an additive effect is assumed. The US EPA (1989) accepts the level of cumulative cancer risk (CCR) between 1×10^{-6} and 1×10^{-4}.

3 Results and Discussion

3.1 Non-cancer Risk

The results of the assessment of non-cancirogenic effects were calculated using the formulas gathered in Tables 4 and 5. The HQ and HI values were calculated for Scenario 1 and Scenario 2 for the averaged exposure level (Table 4) and the maximal exposure level (Table 5). There was no case in which any of the HQ or HI value exceeded the unity threshold. Thus, it may be judged that children were exposed to high enough concentration of pollutants to develop a non-carcinogenic effect in the period of 1986–1997.

3.2 Cancer Risk

The results of the assessment of cancer risk were gathered in Tables 6 and 7. Here, CR and CCR values were calculated for Scenario 1 and Scenario 2 for the averaged exposure level (Table 6) and the maximal exposure level (Table 7). None of the values calculated for nurseries, kindergartens, and schools (Table 6 and 7; Scenario 1) exceeded the defined threshold of 1×10^{-6}. It is worth noting, however, that benzene was responsible for almost 80% of cancer risk at schools, while formaldehyde predominated in nurseries and kindergartens (75%). The average and maximal CR and CCR values did not differ much and indicated relatively small fluctuations in the content of both compounds over the period of time analyzed. The cancer risk calculation for Scenario 2 indicates almost a two-fold greater CR and CCR for both averaged and maximal exposure levels.

Comparison of both presented scenarios indicates that the application of a simplified age-related children risk assessment gives much reduced information that finally may lead to false judgments. This applies mainly to children of 0–1 and 2–3 years of age, where CR indicated no actual carcinogenic effect in Scenario 1, while CR values showed a possible carcinogenic effect in Scenario 2. The situation of overestimation happened in each case of CR evaluation. It should be noted that the greatest differences (almost one order of magnitude) concerned school children and youngsters. In the evaluation of non-carcinogenic effects Scenario 2 presented averaged rather than overestimated values. This

Table 4 Hazard index (HI) and hazard quotient (HQ) for the averaged exposure level

	Scenario 1					Scenario 2	
HQ	0–1	2–3	4–6	7–11	12–16	0–6	7–16
Formaldehyde	1.94E-02	2.21E-02	3.91E-02	1.15E-02	7.12E-03	2.91E-02	8.68E-03
Phenol	2.38E-04	2.70E-04	2.79E-04	1.23E-04	7.61E-05	2.67E-04	9.28E-05
Toluene	1.55E-04	1.76E-04	7.18E-05	5.30E-05	3.27E-05	1.25E-04	3.99E-05
Xylenes (o-. m-. p-)	3.55E-02	4.03E-02	3.87E-03	4.15E-03	2.56E-03	2.30E-02	3.12E-03
Naphthalene			3.99E-03	5.47E-03	3.38E-03		4.12E-03
Benzene	3.18E-03	3.60E-03	1.05E-02	2.07E-02	1.28E-02	6.59E-03	1.56E-02
Butyl alcohol	1.85E-05	2.09E-05	1.04E-05	1.57E-05	9.68E-06	1.52E-05	1.14E-05
HI	**5.85E-02**	**6.64E-02**	**5.78E-02**	**4.20E-02**	**2.59E-02**	**5.90E-02**	**3.16E-02**

Table 5 Hazard index (HI) and hazard quotient (HQ) for the maximal exposure level

	Scenario 1					Scenario 2	
HQ_{max}	0–1	2–3	4–6	7–11	12–16	0–6	7–16
Formaldehyde	2.43E-02	2.76E-02	5.21E-02	1.53E-02	9.44E-03	3.78E-02	1.15E-02
Phenol	3.27E-04	3.72E-04	4.59E-04	1.95E-04	1.84E-04	4.01E-04	1.86E-04
Toluene	1.98E-04	2.24E-04	1.12E-04	8.58E-05	5.30E-05	1.68E-04	6.46E-05
Xylenes (o-. m-. p-)	3.93E-02	4.46E-02	6.82E-03	4.29E-03	2.65E-03	2.66E-02	3.23E-03
Naphthalene			7.98E-03	7.53E-03	4.65E-03		5.67E-03
Benzene	5.16E-03	5.86E-03	1.74E-02	2.20E-02	1.36E-02	1.09E-02	1.66E-02
Butyl alcohol	2.60E-05	2.95E-05	1.29E-05	2.25E-05	1.39E-05	2.06E-05	1.64E-05
HI_{max}	**6.93E-02**	**7.87E-02**	**8.49E-02**	**4.94E-02**	**3.06E-02**	**7.58E-02**	**3.73E-02**

Table 6 Cancer risk (CR) and cumulative cancer risk (CCR) values for the averaged exposure level

	Scenario 1					Scenario 2	
CR	0–1	2–3	4–6	7–11	12–16	0–6	7–16
Formaldehyde	1.07E-11	4.84E-11	1.17E-10	5.74E-11	3.55E-11	2.01E-10	8.65E-11
Benzene	3.20E-12	1.45E-11	5.77E-11	1.89E-10	1.17E-10	8.37E-11	2.85E-10
CCR	**1.39E-11**	**6.29E-11**	**1.75E-10**	**2.47E-10**	**1.52E-10**	**2.85E-10**	**3.72E-10**

Table 7 Cancer risk (CR) and cumulative cancer risk (CCR) values for the maximal exposure level

	Scenario 1					Scenario 2	
CR_{max}	0–1	2–3	4–6	7–11	12–16	0–6	7–16
Formaldehyde	1.33E-11	6.05E-11	1.56E-10	7.62E-11	4.71E-11	2.61E-10	1.15E-10
Benzene	5.20E-12	2.36E-11	9.57E-11	2.02E-10	1.25E-10	1.38E-10	3.04E-10
CCR_{max}	**1.85E-11**	**8.41E-11**	**2.52E-10**	**2.78E-10**	**1.72E-10**	**3.99E-10**	**4.19E-10**

situation imposes an even greater danger as it gives a very shallow knowledge over full characteristics of risk factors experienced by each of age-group. Averaged risk values may ignore situations of actual hazard exposure, although it was not the case encountered in the present study. When place-dependent risk is considered, children in nurseries might be exposed to very low pollutant concentrations, while those in kindergartens might be exposed to greater concentrations. The risk assessment conducted for the age-group 0–6 years indicates risk below the set threshold value. Considering a situation, where only one-age group is exposed to threshold-exceeding risk values and the other is not, a mismanagement of risk-actions could arise, which would unnecessarily consume large amounts of financial resources. Risk assessment, being a multi-step process, is bound to errors that may considerably differ in the nature and magnitude of their consequences. Considering another example, cancer risk assessment in children (e.g., lymphoma) may lead either to false negative or false positive results. Unlike false negative, false positive results are always a matter of further and more relevant studies. False negatives, on the other hand, will result in a false sense of security and may endanger children's life. The schemes outlined above could be avoided by applying a more detailed, shorter age-range stratification for cancer risk assessment in children.

4 Conclusions

Stratification into long age-ranges in the evaluation of children's exposure to hazardous substances is wrong as it could lead to erroneous conclusions concerning the health risk assessment. Due to fast biochemical and physiological changes in children, and differences in the age-related activity patterns, age-related health risk evaluation ought to consist of short age-ranges. The risk assessment indices yield different results in children and adults, causing the use of uniformed assessment procedures highly inefficient and unreliable. Further, even a simplified stratification into just two age-ranges may lead to false judgments on actual risk experienced by children. Such situation may finally lead to either insufficient child health protection or unnecessary financial losses. These encounters cannot be avoided, unless more attention is put to the methodological issues of risk assessment.

Acknowledgements The authors would like to thank Ms. Jolanta Łubkowska and Dr. Renata Wiglusz for an invaluable help in making the resources of HIGMAT Central Data Base available. The study was supported by Grant 2011/01/N/NZ7/01547 from the National Science Center in Poland.

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

Supplementary Material

Table A Age-dependent inhalation rates (m^3/h)

	Scenario 1					Scenario 2	
	Age-group (years)					Age-group (years)	
Activity	0–1	2–3	4–6	7–11	12–16	0–6	7–16
Sleep/nap	3.00E-03	4.55E-03	4.30E-03	4.50E-03	5.00E-03	3.95E-03	4.75E-03
Sedentary/passive	3.10E-03	4.75E-03	4.50E-03	4.80E-03	5.40E-03	4.12E-03	5.10E-03
Light intensity	7.60E-03	1.20E-02	1.10E-02	1.10E-02	1.30E-02	1.02E-02	1.20E-02
Moderate intensity	1.40E-02	2.10E-02	2.10E-02	2.20E-02	2.50E-02	1.87E-02	2.35E-02
High intensity	2.60E-02	3.85E-02	3.70E-02	4.20E-02	4.90E-02	3.38E-02	4.55E-02

Table B Percentage of age-dependent duration (hours/24h) of performing specific activity

	Scenario 1					Scenario 2	
	Age-group (years)					Age-group (years)	
Activity (h/24h) (%)	0–1	2–3	4–6	7–11	12–16	0–6	7–16
Sleep/nap	55.2%	51.4%	46.5%	42.6%	39.5%	51.0%	41.0%
Sedentary/passive	5.3%	9.1%	12.7%	14.7%	18.9%	9.0%	16.8%
Light intensity	23.5%	23.6%	26.8%	31.0%	31.4%	24.7%	31.2%
Moderate intensity	15.8%	15.8%	13.5%	10.9%	9.1%	15.0%	1.0%
High intensity	0.8%	0.8%	1.0%	1.2%	1.4%	0.9%	1.3%

Table C Children's body mass

	Scenario 1					Scenario 2	
	Age-group (years)					Age-group (years)	
	0–1	2–3	4–6	7–11	12–16	0–6	7–16
Average body mass (kg)	9.1	12.3	17.5	28.7	52.3	13.0	40.5

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 33: 55–62
DOI 10.1007/5584_2017_28
© Springer International Publishing Switzerland 2017
Published online: 6 April 2017

Metabolic Syndrome as a Factor Affecting Systemic Inflammation in Patients with Chronic Obstructive Pulmonary Disease

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Abstract

Chronic obstructive pulmonary disease (COPD) is a systemic disease which may be associated with other comorbidities. The aim of the study was to estimate the incidence of metabolic syndrome (MS) in COPD patients and to assess its impact on systemic inflammation and lung function. MS was diagnosed in accordance with the recommendations of the Polish Forum for the Prevention of Cardiovascular Diseases. The study group consisted of 267 patients with stable COPD in all stages of severity. All patients underwent spirometry with bronchial reversibility testing and 6 min walk test (6MWT). The following blood tests were evaluated: lipid profile, glucose and C-reactive protein as well as serum concentration of IL-6, leptin, adiponectin, and endothelin. MS was diagnosed in 93 patients (35.8%). No differences were observed in the incidence of MS in relation to airflow limitation severity (mild; moderate; severe and very severe: 38.9; 36.3; 35.2 and 25.0%, respectively). FEV_1 (% predicted), FVC (% predicted), 6MWT distance (6MWD), age, and the number of pack-years were similar in patients with and without MS. MS was more frequent in males than females (38.7 vs. 28.4%, $p > 0.05$). Serum concentrations of IL-6, endothelin, leptin, and CRP were higher in

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the MS group, contrary to adiponectin concentration which was lower ($p < 0.01$). MS was more frequent in male COPD patients, but there were no differences in its frequency between patients with different severity of airflow limitation. We conclude that MS, as a comorbidity, occurs in all COPD stages and affects systemic inflammation. MS incidence does not depend on COPD severity.

Keywords
Adiponectin • Bronchial reversibility test • Comorbidities • Endothelin • Interleukin 6 • Lung disease • Spirometry

1 Introduction

Chronic obstructive pulmonary disease (COPD) has been long recognized as a systemic disease leading to complications and enhancing the development or aggravating numerous other diseases. It is estimated that only 6% of COPD patients are free from other chronic health-related problems, and that over 50% of patients have one or two coexisting conditions, 17% have three or four conditions, and 7% have five or more concurrent diseases (van Manen et al. 2001). The most significant co-morbid conditions are the following: malnutrition and muscular strength reduction, cardiovascular diseases, osteoporosis, metabolic disorders, anxiety, and depression (GOLD 2016). Metabolic syndrome (MS), defined as hyperinsulinaemia and presence of at least two of the following factors: obesity, arterial hypertension, dyslipidemia, and glucose level disorders occurs in approximately 15% of the non-diabetic European population (Hu et al. 2004). Tobacco smoking is a common risk factor for both COPD and metabolic syndrome. Park et al. (2003) demonstrated that current smoking was a significant risk factor for metabolic syndrome in males. On the other hand, abdominal obesity has an effect on the course of respiratory diseases (Lam et al. 2010; Leone et al. 2009; Guerra et al. 2002). Both COPD and metabolic syndrome were demonstrated to be associated with increased systemic inflammation (increased levels of C reactive protein, tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6)) (van den Borst et al. 2012; Karadag et al. 2008). Adipose tissue also is a source of proteins participating in systemic inflammation such as leptin and adiponectin (Stylianou et al. 2007). A common feature of COPD and metabolic syndrome is co-existence with cardiovascular disordersand increased related mortality (Corbaton-Anchuelo et al. 2013; Ghoorah et al. 2013; Barnes and Celli 2009). MS definition has been changed several times over recent years, starting from the WHO description formulated in 1998 (Alberti and Zimmet 1998), through the third report of the National Cholesterol Education Program (NCEP 2001), the criteria of the International Diabetes Federation (2006), to the guidelines of the Polish Forum for the Prevention of Cardiovascular Diseases published in 2009 (Mamcarz et al. 2009).

The aim of the present study was to estimate the incidences of MS in COPD patients and to assess the influence of MS on systemic inflammation and lung function.

2 Methods

2.1 Patients and Protocol

The study was approved by the Bioethics Committee of Warsaw Medical University in Poland (permit # KB207/2008). The study group

Table 1 Criteria for the diagnosis of metabolic syndrome of the Polish Forum for the Prevention of Cardiovascular Diseases (Mamcarz et al. 2009)

Central obesity	Waist circumference: males ≥94 cm; females ≥80 cm
Triglycerides	≥150 mg/dL (≥1.7 mmol/L) or specific treatment for this abnormality (fibrates, nicotinamide, or high dose of omega-3 fatty acids)
HDL cholesterol	Males: <40 mg/dL (<1.04 mmol/L); females: <50 mg/dL (<1.3 mmol/L) or specific treatment for this abnormality (fibrates or nicotinamide)
Arterial blood pressure	Systolic ≥130 mmHg and/or diastolic ≥85 mmHg or specific treatment for previously diagnosed arterial hypertension
Fasting plasma glucose	≥100 mg/dL (5.6 mmol/L) or specific treatment for this abnormality

Metabolic syndrome is diagnosed when at least three of the criteria below outlined are met

included 267 consecutive patients with stable COPD (F/M; 98/169, mean age 65.6 ± 9.3 yr) in all stages of the severity of airflow limitation: mild: n = 18 (6.8%); moderate: n = 113 (42.3%); severe: n = 108 (40.4%), and very severe: n = 28 (10.5%). The enrolled patients were treated on an out-patient basis at the Department of Internal Medicine, Pneumology and Allergology of Warsaw Medical University in Poland. The inclusion criteria were: COPD diagnosis and signed informed consent. The exclusion criterium was an exacerbation within 4 weeks prior to enrollment.

The collected data included the history of co-morbidities and medicines used, basic anthropometry such as body weight, height, waist and hip circumference, and the arterial blood pressure measurement. All these examinations were performed before breakfast, after overnight bedrest. Body mass index BMI (kg/m^2) and waist-to-hip ratio were calculated. Spirometry with bronchial reversibility testing after administration of 400 μg of salbutamol *via* a spacer was performed using a Lungtest 1,000 device (MES, Cracow, Poland) according to the recommendations of the ATS/ERS (Miller et al. 2005). In 172 subjects, 6 min walk test (6MWT) was performed according to the guidelines of the American Thoracic Society (ATS 2002). MS was diagnosed according to the recommendations of the Polish Forum for the Prevention of Cardiovascular Diseases (Mamcarz et al. 2009) (Table 1).

2.2 Biomarkers

Fasting blood samples were drawn for the content of serum lipids, glucose, and also for interleukin-6 (IL-6), adiponectin, leptin, endothelin (ET-1), insulin, resistin, and insulin-like growth factor 1 (IGF-1). Serum lipids and glucose were measured in the central hospital laboratory using routine methods. Insulin was assessed using the enzyme-linked immunosorbent assay (ELISA) (Phoenix Pharmaceuticals Inc., Burlingame, CA). The other variables were assessed using Quantikinine ELISA kits (R&D Systems; Minneapolis, MN).

2.3 Statistical Elaboration

Data were presented as medians and interquartile ranges (IQR). The Shapiro-Wilk test was used for the estimation of data distribution. As the majority of variables demonstrated non-normal distribution, we applied the Mann-Whitney U and Kruskal-Wallis rank tests for group comparisons. Correlations were evaluated using Spearman's rank correlation test. A p-value <0.05 defined statistically significant differences. The evaluation was performed using a commercial statistical package of Statistica v10 for Windows (Statsoft Inc.; Tulsa OK).

Table 2 Clinical features of patients with chronic obstructive pulmonary disease (COPD)

Variable	Median (IQR)
Pack-years	38.4 (22.3–50.0)
BMI (kg/m^2)	27.9 (23.0–31.5)
Waist circumference (cm)	100 (88–108)
FEV_1 (L)	1.4 (1.0–1.8)
FVC (L)	53.1 (29.6–66.2)
FEV_1 (% predicted)	2.9 (2.1–3.5)
FVC (% predicted)	81.2 (65.5–95.2)
COPD co-morbidities	Number of patients (%)
Ischemic heart disease	87 (31.6)
Myocardial infarction	32 (11.6)
Arterial hypertension	179 (65.1)
Diabetes mellitus	60 (21.8)
Stroke	13 (4.7)

3 Results

Clinical features of COPD patients are displayed in Table 2. MS was diagnosed in 93 patients (34.8%). There were no differences in the MS incidence regarding the degree of COPD-related airflow limitation. MS was diagnosed in 38.9% patients with mild; 36.3% with moderate; 35.2% with severe, and 25.0% with very severe airflow limitation. It was diagnosed in 28.4% of female and 38.7% of male patients ($p > 0.05$). There were no appreciable differences in age and pack-years of cigarette smoking between patients with and without MS. The BMI was greater in the MS group (30.5 kg/m^2, IQR: 26.3–34.8 kg/m^2) compared to COPD patients without MS (26 kg/m^2, IQR: 22.0–29.6 kg/m^2, $p < 0.0001$). Likewise, waist circumference was greater in the MS than non-MS group (105.0 cm, IQR: 101–114 cm vs. 91.0 cm, IQR: 85–102 cm, respectively; $p < 0.0001$). Arterial hypertension was more frequent in the MS than non-MS group (86% vs. 14%, respectively; $p < 0.001$), as was also diabetes mellitus (56% vs. 44% respectively; $p < 0.05$). There were no differences in the prevalence of ischemic heart disease (28% vs. 38%) or stroke (6% vs. 3%) between the MS vs. non-MS group, respectively. Nor were there any differences in spirometry and 6MWD between the two groups of COPD patients. However, patients with MS had higher systolic blood pressure recorded after the 6MWT. A compilation of the results outlined above is displayed in Table 3.

The median (IQR) serum concentrations of IL-6, leptin, and ET-1 were higher in the MS(+) than in MS (−) group: 4.7 (2.4–7.4) pg/mL vs. 2.8 (2.0–4.1) pg/mL, ($p = 0.004$); 14.5 (9.9–29.3) μg/L vs. 10.7 (5.1–19.0) μg/L, ($p = 0.008$), and 1.9 (1.8–2.5) pg/mL vs. 1.8 (1.5–2.1) pg/mL, ($p = 0.007$). Adiponectin was lower in the MS(+) than in MS(+) group: 6.7 (5.0–9.8) mg/L vs. 10.9 (7.1–19.4) mg/L, ($p < 0.0001$) (Fig. 1). CRP was higher in the MS (+) than in MS(−) group: 4.6 (2.5–8.1) mg/L vs. 2.5 (2.4–6.3) mg/L, ($p = 0.002$). The remaining biochemical indices studied were not different between the COPD patients with and without MS (Table 4).

4 Discussion

In the present study, MS was found in 34.9% of subjects, more frequently in men compared to women, but the difference was not statistically significant. We failed to find any appreciable differences in the MS incidence according to the airflow limitation severity. Studies performed in other cohorts show some differences. Díez-Manglano et al. (2014) have found the MS in 42.9% of their subjects, with female predominance. Similar to the present study, no effect of age or smoking status on the prevalence rate was demonstrated. In a study by Watz et al. (2009), MS was diagnosed in more than 50% patients with mild and moderate airflow limitation, MS frequency was comparable to that observed in chronic bronchitis, and it was associated with increased content of systemic inflammation markers. In the Japanese population, there is no difference in the occurrence of MS between COPD patients and healthy individuals. There is, however, a difference depending on the severity of COPD; MS is present in 16.8% of the patients in Stage I vs. 28.7% in Stage II-IV of the disease (Funakoshi et al. 2010).

The present study confirmed a pro-inflammatory effect of MS. Serum

Table 3 Pulmonary function and 6 min walking test (6MWT) in patients with chronic obstructive pulmonary disease (COPD) with and without accompanying metabolic syndrome (MS(+) and MS(−))

Variable	MS (−)	MS (+)	p
FEV_1 (% predicted)	49.7 (38.9–67.0)	50.4 (44.5–65.2)	ns
FVC (% predicted)	81.7 (65.9–96.5)	80.0 (65.4–94.4)	ns
6MWD (m)	392 (300–480)	391 (320–470)	ns
6MWT – baseline before test			
Heart rate, min^{-1}	81 (73–89)	81 (72–89)	ns
Systolic blood pressure, mmHg	119 (110–130)	127 (120–140)	0.001
Diastolic blood pressure, mmHg	72 (65–80)	75 (70–80)	0.05
6MWT – end of test after 6 min			
Heart rate, min^{-1}	101 (90–110)	101 (89–110)	ns
Systolic blood pressure, mmHg	136 (125–145)	147 (130–160)	<0.001
Diastolic blood pressure, mmHg	76 (70–80)	78 (70–85)	ns

Data are medians and interquartile ranges (IQR); *FEV_1* forced expiratory volume in 1 s, *FVC* forced vital capacity, *6MWD* six minute walking distance, *ns* non-significant

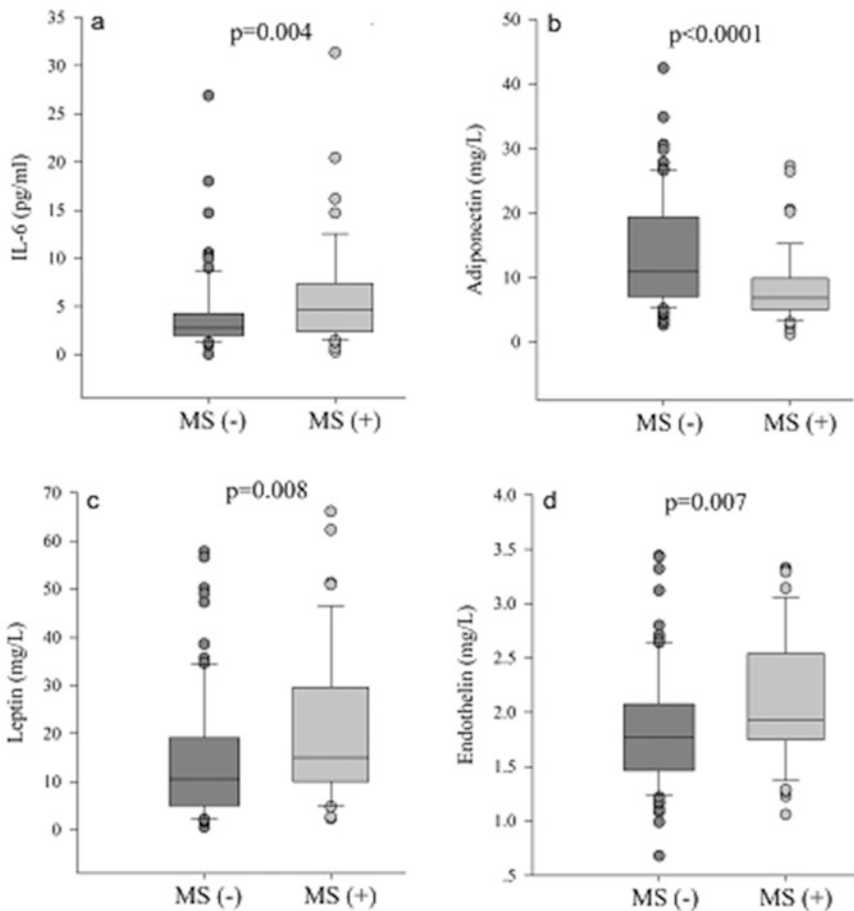

Fig. 1 Differences in (**a**) interleukin-6 (IL-6); (**b**) adiponectin; (**c**) leptin, and (**d**) endothelin) between COPD patients with (MS (+)) and without metabolic syndrome (MS (−)). The *horizontal line* within the *box* indicates the median. The boundaries of the *box* indicate the 25th and 75th percentile. The *error bars* mark the 10th and 90th percentiles. *Dotted points* represent the outlying values

Table 4 Biochemical indices in chronic obstructive pulmonary disease (COPD) patients with (MS(+)) and without (MS(−)) metabolic syndrome

Variable	MS (−)	MS (+)	p
Resistin (ng/mL)	7.6 (6.4–9.5)	8.7 (6.9–10.4)	ns
Insulin (U/L)	0.6 (0.3–1.7)	1.5 (0.7–4.2)	ns
IGF-I (ng/mL)	102 (88–130)	102 (85–117)	ns

IGF-1, insulin-like growth factor 1; *ns* – not significant

adiponectin, a protein reducing insulin resistance and having anti-inflammatory and anti-athermanous effects was significantly lower in MS than that in the group of COPD patients without MS. Serum content of pro-inflammatory proteins, on the other hand, such as IL-6, leptin, and CRP, was greater in MS patients as also was ET-1, an aminopeptide having a contractive effect on both blood vessels and bronchi. In a study by Bae et al. (2013) adiponectin, TNF-α, and hsCRP were the most proximate markers reflecting MS, which is in line with other studies on the subject (Akpinar et al. 2012; Poulain et al. 2008). Serum ET-1 concentration in bronchoalveolar lavage fluid from smokers has been found higher compared to non-smokers (Reichenberger et al. 2001), higher in COPD patients compared to healthy controls (Bacakoglu et al. 2003), and also higher in obese MS patients (Ferri et al. 1997). The MS has an enhancing effect on serum ET-1, which further confirms the role of MS in the pathologies associated with epithelial dysfunction, COPD patients inclusive. In the present study we did not observe any differences in the frequency of MS in patients with different airflow limitation severity. Nor were there any appreciable differences in the results of FVC and 6 min walking test between patients with and without MS. That is at variance with some other studies where differences in lung function between COPD patients with and without MS are reported. Korean researchers have shown that both women and men with MS have lower FVC (% predicted) and a lower FEV_1%FVC. Those authors show an inverse association between FVC and waist circumference or arterial blood pressure (Choi et al. 2011). The analysis of 547 studies have confirmed an inverse association between waist circumference and pulmonary function; the association was particularly evident in men (Wehrmeister et al. 2012). Further, an effect of MS on the annual decline of FEV_1 has also been observed (Sato et al. 2013). Lung function impairment is associated with MS independently of age, sex, smoking status, alcohol consumption, educational level, body mass index, cardiovascular disease history and others (Leone et al. 2009). Minas et al. (2011) have proposed a specific COPD phenotype characterized by a relatively young age, moderate impairment of pulmonary function, insulin resistance, and leptin-adiponectin imbalance. Patients with this phenotype would require special attention as they have a less favorable course of COPD and increased risk of extrapulmonary complications, particularly cardiovascular diseases, and increased mortality.

The findings of the present study are limited by the lack of a control group of healthy smokers, in whom the MS occurrence rate were assessed. Nonetheless, we believe that the findings, along with the literature data, confirm that the identification of patients with the MS phenotype and the introduction of appropriate preventive measures may improve prognosis and reduce mortality rate in COPD patients.

In conclusion, this study demonstrates that metabolic syndrome is one of the pathologic conditions accompanying COPD and may develop at any stage of the disease. We found no differences in the MS incidence in COPD patients depending on the severity of airflow limitation, but systemic inflammation activity and body mass index were higher in the MS group. The findings suggest that screening for the metabolic syndrome ought to be part of a routine evaluation for the presence of diseases accompanying COPD.

Acknowledgements The study was performed as the part of National Center for Research and Development project 'Chronic obstructive pulmonary disease (COPD) – systemic disease; the biggest threat of twenty-first century' (NR 13 0034 06/2009).

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

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 33: 63–72
DOI 10.1007/5584_2017_29
© Springer International Publishing Switzerland 2017
Published online: 30 April 2017

Carotid Intima-Media Thickness and Metabolic Syndrome Components in Obese Children and Adolescents

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Abstract

Obesity in children and adolescents contributes to increased prevalence of metabolic and hemodynamic complications, which may impair endothelial function and structure. A high resolution B-mode ultrasound measurement of intima-media thickness (IMT) is a useful tool to assess early, preclinical stage of atherosclerosis. The objective of this study was to evaluate the carotid artery IMT in obese children and its association with insulin resistance and other traditional metabolic syndrome components. The study entailed 80 obese children, aged 5.3–17.9 year and a control group of 31 children. Obesity was defined using the International Obesity Task Force (IOTF) criteria. Metabolic syndrome was defined using the International Diabetes Federation (IDF) criteria of 2007. Each patient's anthropometric measurements, blood parameters, and the carotid IMT were evaluated. Insulin resistance indices were calculated. We found that children with metabolic syndrome had a significantly increased IMT compared to children who did not meet the syndrome criteria (0.62 ± 0.09 mm *vs.* 0.55 ± 0.18 mm, $p = 0.03$) and compared to control group (0.62 ± 0.09 *vs.* 0.52 ± 0.14, $p = 0.02$). In a multivariable linear regression analysis, IMT correlated with systolic blood pressure ($p = 0.005$). The results did not show an association between IMT and insulin resistance. We conclude that abdominal obesity and the accompanying components of metabolic syndrome lead to increased

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carotid IMT. The enhanced systolic blood pressure plays a major role in changing the carotid IMT.

Keywords
Blood pressure • Carotid artery • Intima–media thickness • Metabolic syndrome • Obesity

1 Introduction

Atherosclerotic process and fibrous plague formation are the most frequent causes of mortality worldwide. Atherosclerosis does not usually provoke any symptoms even for decades until a sudden onset of coronary events such as stroke, myocardial infarction, or peripheral vascular disease. It is well-known that obesity in children and adolescents can induce atherosclerotic vascular changes even at an early age. Furthermore, fat mass accumulation (mainly visceral) favors the occurrence of metabolic and hemodynamic disturbances, whose severity and coexistence also play a crucial role in the pathogenesis of atherosclerotic process (Hong 2010; Berenson et al. 1998). The risk of its occurrence increases with increasing body mass index (BMI). At least two risk factors for cardiovascular disease were found in 39% of children and adolescents with BMI $\geq$ 95th percentile (pc), and in 59% of those with BMI $\geq$ 99 percentile (Freedman et al. 2007).

Therefore, it is important to take action to promote a healthy lifestyle and to identify patients who are at a risk of developing atherosclerosis. Besides traditional risk factors for coronary artery disease (CAD), the measurement of intima-media thickness (IMT) of the carotid artery using high-resolution ultrasonography has been used to evaluate early changes in the endothelium structure, which reflect the severity and extent of atherosclerosis in other arteries. The IMT is a useful surrogate marker of subclinical atherosclerosis in a wide spectrum of age (Johnson et al. 2009). In adults, IMT correlates with severity and extent of CAD such as myocardial infarction and stroke. Increased IMT precedes the occurrence of cardiovascular events, irrespective of traditional risk factors (O'Leary et al. 1999). In children, increased carotid IMT has been found in patients with familiar hypercholesterolemia (Vijayasarathi and Goldberg 2014), primary hypertension (Bucher et al. 2013), chronic kidney disease (Brady et al. 2012), and type 1 diabetes (Rodriguez et al. 2007). A large body of evidence indicates that adiposity and obesity-related metabolic and hemodynamic disturbances adversely affect the endothelium. The results concerning the association between IMT and cardiometabolic risk factors in obese children vary and are not fully consistent (Elshorbagy et al. 2016; Fang et al. 2010; Giannini et al. 2008; Reinehr et al. 2006). Therefore, this study seeks to determine the IMT in obese children and adolescents and the IMT's association with insulin resistance and other traditional components of metabolic syndrome.

2 Methods

The study included 80 obese children (50 boys and 30 girls) aged 5.3–17.9 years (mean age 11.4 $\pm$ 2.9 years) hospitalized at the Department of Pediatrics and Endocrinology of the Medical University of Warsaw in Poland. After excluding endocrine, hereditary, kidney and infectious diseases, obesity was defined using the BMI cutoff level for children according to the International Obesity Task Force (IOTF) (Cole et al. 2000). The metabolic syndrome (MetS) criteria for obesity in children aged $\geq$10 years

were adopted according to the International Diabetes Federation (IDF) Consensus Statement (Zimmet et al. 2007). Hence, examined subjects were divided by age into two subgroups: children younger than 10 years and older than 10 years. The older children were further divided according to the presence or absence of MetS. The controls were 31 healthy non-obese children (mean age 13.1 ± 2.6 years). The study participants underwent physical examination, anthropometric measurements, blood tests, and their carotid intima media thickness was evaluated by ultrasonography. The study protocol was approved by the Bioethics Committee of the Medical University of Warsaw in Poland.

The anthropometric measurements consisted of body height (cm), body weight (kg), waist and hip circumference (cm), and thickness of skinfolds under the triceps brachii muscle and under the inferior scapular angle (mm). The results of these measurements were used to determine BMI, waist to hip ratio (WHR), and waist to height ratio (WHtR). The degree of obesity (expressed as SDS BMI) was quantified using the least mean square method of Cole (1990). Body fat percentage (FAT) was calculated using the Slaughter equations based on skinfold measurements (Slaughter et al. 1998) and additionally in obese children using the bioelectrical impedance analysis (BIA) (Body Fat Analyzer BF-905; Maltron International Ltd., Rayleigh, UK).

After a 12-h overnight fast, blood samples were taken to measure fasting glucose and lipid profile (total cholesterol - TC, high density lipoprotein cholesterol - HDL-C, triglycerides - TG) using a standard enzymatic method. Light-density lipoprotein cholesterol (LDL-C) was calculated using Friedewald's formula. The oral glucose tolerance test (OGTT) was performed in obese children, but not in healthy controls. The obtained data were used to calculate indicators of insulin resistance: homoeostasis model assessment (HOMA), fasting glucose to insulin ratio (FGIR), quantitative insulin sensitivity check index (QUICKI), oral glucose insulin sensitivity index (OGIS), and Matsuda indices (Ten and Maclaren 2004). Hyperlipidemia was interpreted according to the American Heart Association (AHA) recommendations (Hayman et al. 2007). Glucose levels were interpreted in accordance with the Polish Diabetes Association guidelines (PDA 2016). Severe insulin resistance was defined as the HOMA value $\geq$3.

In obese children, blood pressure (BP) was measured using a sphygmomanometer. After 10 min of rest, BP was measured three times and the average was taken for analysis. Hypertension was diagnosed when the values of systolic (SBP) or diastolic blood pressure (DBP) were above the 95th percentile for the Polish population of children; mean blood pressure values between the 90th and 95th percentiles were defined as the border zone (Ostrowska-Nawarycz and Nawarycz 2007).

Carotid IMT was measured by a trained investigator using 12–15 MHz ultrasound high resolution linear vascular probe on ATL 3000 HDL (Bothell; Washington DC). IMT measurements were assessed three times on both sides at the far wall of common carotid artery about 1 cm from the bifurcation as the distance between the two parallel echogenic lines: between lumen-intima and media-adventitia interferences. The average of these measurements was considered as the final carotid IMT value.

Data were presented as means ± SD, and minimum and maximum values, unless otherwise indicated. Differences between study groups were analyzed by independent samples *t*-test or Mann-Whitney U test. Associations between carotid IMT and chosen anthropometric, biochemical variables, and blood pressure were determined by Spearman's nonparametric correlation. Multivariable linear regression analysis was used to quantify the influence of chosen anthropometric parameters, metabolic factors, and blood pressure on IMT. A p-value <0.05 was assumed to define statistical significance of differences. Statistical analysis was performed using a commercial IBM SPSS 19 statistical packet (Armonk, NY).

3 Results

The mean BMI of the obese children was 29.2 ± 4.6 kg/m^2, the mean BMI SDS was 2.8 ± 0.5. For most anthropometric parameters and lipids profile there were statistically significant differences between the study group and the control group (Table 1). Increased values of total cholesterol (TC ≥ 200 mg/dl) were found in 20.0% of obese children, raised low-density lipoprotein cholesterol concentrations (LDL-C ≥ 110 mg/dl) was detected in 41.3% of obese children. Triglycerides (TG) levels exceeding 110 mg% were observed in 52 obese children (65.0% of the group). Low levels of high-density lipoprotein cholesterol (HDL-C < 40 mg/dl) were found in 28 (35.0%) obese children. Impaired fasting glucose was diagnosed in 9 (11.3%) and impaired glucose tolerance in 22 (27.5%) obese children. Elevated fasting insulin levels ≥15 μIU/ml were found in 47.5% of the children. The mean value of the insulin resistance indicator HOMA was 3.4 ± 2.1, whereas 52.5% of the group had HOMA values showing severe insulin resistance ≥3. The percentage of obese children with SBP slightly above the 95th percentile was 7.5%, and for DBP the percentage was 17.5%. The anthropometric and laboratory characteristics for obese children disaggregated by age and the presence of MetS are presented in Table 2.

The MetS criteria were applied to obese children at the age of 10 years and older according to the International Diabetes Federation recommendations. Among the 54 obese children, 42 (77.8%), including 16 girls and 26 boys, had central obesity, 20 (37.0%) had elevated levels of TG (≥150 mg/dl), and 20 children (37.0%) had decreased levels of HDL-C (< 40 mg/dl). In 10 children (10.0%) increased levels of SBP (≥130 mg/dl) or decreased levels of DBP (< 85 mmHg) were found. Five obese children (9.3%) had impaired fasting glucose (≥100 mg/dl). Diagnosis of metabolic syndrome (presence of central obesity plus any two or more out of other four components) was made found in 12 (22.2%) obese patients. The MetS (+) and the MetS (−) subgroups did not demonstrate significant differences regarding anthropometric parameters describing obesity and insulin resistance indices, as shown in Table 2.

The mean carotid IMT measurement did not show significant differences between obese children and the normal-weight control group (0.55 ± 0.15 mm *vs*. 0.52 ± 0.14 mm; p = 0.4). When we compared the subgroup of obese

Table 1 Anthropometric and biochemical parameters in obese and control non-obese children

Variable	Obese children (n = 80)	Non-obese children (n = 31)
Height (cm)	153.1 ± 16.1	156.9 ± 13.1
Body weight (kg)	70.4 ± 22.0***	45.3 ± 12.8
BMI (kg/m^2)	29.2 ± 4.6***	18.2 ± 2.7
SDS BMI	2.77 ± 0.46***	−0.19 ± 0.92
WC (cm)	89.3 ± 11.3***	63.4 ± 6.6
HC (cm)	100.5 ± 13.3***	81.4 ± 10.1
WHR	0.89 ± 0.06***	0.78 ± 0.05
WHtR	0.58 ± 0.05***	0.40 ± 0.02
% FAT (skinfold)	33.7 ± 4.4***	18.5 ± 5.9
Fasting glucose (mg/dl)	84.7 ± 10.5	81.3 ± 10.2
TC (mg/dl)	177.2 ± 30.9**	159.6 ± 22.9
HDL-C (mg/dl)	44.6 ± 11.4 ***	56.3 ± 12.4
LDL-C (mg/dl)	105.6 ± 27.5 **	87.6 ± 26.0
TG (mg/dl)	134.4 ± 55.0***	77.8 ± 36.0
IMT (mm)	0.55 ± 0.15	0.52 ± 0.14

Data are means ±SD; *BMI*body mass index, *WC* waist circumference, *HC* hip circumference, *WHR* waist to hip ratio, *WHtR* waist to height ratio, *% FAT* % of body mass, *TC* total cholesterol, *TG* triglycerides, *IMT* intima media thickness, ***p < 0.001, **p < 0.01

children aged 10 years and older with control individuals of similar mean age, the differences in the mean value of IMT were somehow bigger (0.57 ± 0.17 *vs*. 0.52 ± 0.14; p = 0.2) and further increased for children with metabolic syndrome (0.62 ± 0.09 *vs*. 0.52 ± 0.14; p = 0.02) (Fig. 1). Carotid IMT did not differ significantly between obese girls and boys (0.58 ± 0.19 mm *vs*. 0.53 ± 0.11 mm; p = 0.17). Children aged 10 years and older had a slightly higher mean value of carotid IMT than younger children (0.57 ± 0.17 *vs*. 0.53 ± 0.10; p = 0.13). Higher carotid IMT values were found in children who met the IDF criteria of metabolic syndrome compared to those who did not have MetS diagnosis (0.62 ± 0.09 mm *vs*. 0.55 ± 0.18 mm; p = 0.03).

In the correlation analysis between carotid IMT and anthropometric parameters assessing

Table 2 Anthropometric and biochemical parameters, blood pressure, and intima media thickness in the obese children divided according to age and the presence of metabolic syndrome

Variable	Obese children (n = 80)	Obese children <10 years (n = 26)	Obese children ≥10 years		
			Total (n = 54)	MetS (−) (n = 42)	MetS (+) (n = 12)
Age (years)	11.4 ± 2.9	8.1 ± 1.3	13.1 ± 1.9***	12.8 ± 1.8	13.9 ± 2.1
Height (cm)	153.1 ± 16.1	135.3 ± 9.7	161.7 ± 10.5***	160.9 ± 11.0	164.5 ± 8.1
Body weight (kg)	70.4 ± 22.1	47.6 ± 9.5	81.4 ± 17.3***	80.4 ± 19.2	85.1 ± 7.7
BMI (kg/m^2)	29.2 ± 4.6	25.7 ± 2.5	30.8 ± 4.4***	30.6 ± 4.8	31.5 ± 2.8
SDS BMI	2.77 ± 0.46	3.02 ± 0.47	2.65 ± 0.41**	2.64 ± 0.44	2.66 ± 0.33
WC (cm)	89.3 ± 11.3	78.8 ± 6.6	94.3 ± 9.5***	93.5 ± 10.3	97.3 ± 4.9
HC (cm)	100.5 ± 13.3	86.9 ± 7.0	107.1 ± 10.4***	106.6 ± 11.4	108.9 ± 5.2
WHR	0.89 ± 0.06	0.9 ± 0.04	0.88 ± 0.06	0.88 ± 0.07	0.90 ± 0.04
WHtR	0.58 ± 0.05	0.6 ± 0.04	0.58 ± 0.05	0.58 ± 0.05	0.59 ± 0.04
%FAT (skinfold)	33.7 ± 4.4	32.3 ± 3.3	34.4 ± 4.8*	34.2 ± 4.6	34.9 ± 5.7
%FAT (BIA)	37.8 ± 7.7	34.5 ± 6.3	39.4 ± 7.8**	39.5 ± 7.1	39.2 ± 10.5
Fasting glucose (mg/dl)	84.7 ± 10.5	84.2 ± 10.7	84.9 ± 10.6	84.0 ± 10.2	88.1 ± 11.5
Fasting insulin (μIU/ml)	16.0 ± 9.0	12.9 ± 6.8	17.4 ± 9.6*	16.5 ± 9.5	20.6 ± 9.5
HOMA	3.40 ± 2.09	2.72 ± 1.54	3.71 ± 2.24*	3.45 ± 2.09	4.64 ± 2.59
MATSUDA	3.91 ± 2.91	5.46 ± 4.26	3.19 ± 1.59*	3.37 ± 1.67	2.54 ± 1.15
OGIS	425.1 ± 81.7	467.7 ± 91.0	405.3 ± 69.4**	409.8 ± 66.8	389.8 ± 79.0
FGIR	6.71 ± 3.30	8.38 ± 4.18	5.94 ± 2.48*	6.23 ± 2.62	4.93 ± 1.67
QUICKI	0.14 ± 0.01	0.15 ± 0.01	0.14 ± 0.01*	0.14 ± 0.01	0.14 ± 0.01
TC (mg/dl)	177.2 ± 30.9	179.7 ± 24.1	176.0 ± 33.8	174.8 ± 33.9	180.0 ± 34.6
HDL-C (mg/dl)	44.6 ± 11.4	49.8 ± 14.8	42.1 ± 8.4*	44.2 ± 7.9	34.8 ± 5.5***
LDL-C (mg/dl)	105.6 ± 27.5	105.0 ± 23.5	105.8 ± 29.4	104.6 ± 28.9	110.1 ± 32.0
TG (mg/dl)	134.4 ± 55.0	123.7 ± 53.5	139.5 ± 55.4	129.2 ± 51.3	175.5 ± 56.2**
SBP (mmHg)	114.7 ± 10.1	109.1 ± 6.6	117.4 ± 10.5**	115.0 ± 9.6	125.0 ± 9.7**
DBP (mmHg)	72.0 ± 8.9	69.7 ± 6.5	73.9 ± 9.6	73.1 ± 9.0	76.4 ± 11.3
IMT (mm)	0.55 ± 0.15	0.53 ± 0.10	0.57 ± 0.17	0.55 ± 0.18	0.62 ± 0.09*

Data are means ±SD; *BMI* body mass index, *WC* waist circumference, *HC* hip circumference, *WHR* waist to hip ratio, *WHtR* waist to height ratio, *% FAT* % of body mass, *TC* total cholesterol, *TG* triglycerides, *SDB* systolic blood pressure, *DBP* diastolic blood pressure, *IMT* intima media thickness, *MetS* metabolic syndrome, *HOMA* homoeostasis model assessment, *FGIR* fasting glucose to insulin ratio, *QUICKI* quantitative insulin sensitivity check index, *OGIS* oral glucose insulin sensitivity index, *MATSUDA* insulin sensitivity indices, ***p < 0.001, **p < 0.01, *p < 0.05 (comparisons: obese children <10 years *vs*. obese ≥10 years, obese children with MetS *vs*. obese children without MetS)

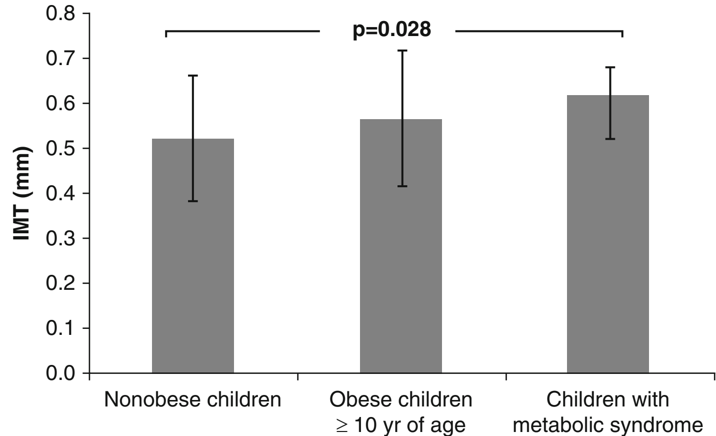

Fig. 1 Comparison of intima media thickness (IMT) between control non-obese children, obese children aged over 10 years, and obese children fulfilling the metabolic syndrome criteria. Data are means ±SD

Table 3 Multivariate regression analysis evaluating correlations between intima media thickness (IMT) and anthropometric, metabolic, and hemodynamic parameters

Independent Variables	*β*	SE	p-value
Age	−0.015	0.014	0.284
WC	0.002	0.003	0.284
SDS BMI	−0.005	0.061	0.616
Fasting glucose	0.002	0.002	0.302
HDL-C	0.001	0.002	0.952
LDL-c	−0.001	0.001	0.392
SBP	0.007	0.002	0.005
DBP	0.001	0.003	0.912

WC waist circumference, *SDS BMI* standard deviation score body mass index, *HDL-C* HDL cholesterol, *LDL-C* LDL cholesterol, *SBP* systolic blood pressure, *SBP* diastolic blood pressure, *β* non-standardized coefficient, *SE* standard error

nutritional status, biochemical parameters, and blood pressure it was found that IMT correlated positively with glucose at 90 min ($r = 0.26$, $p = 0.02$) and 120 min during OGTT ($r = 0.23$, $p = 0.04$), and also with systolic (SBP) ($r = 0.30$; $p = 0.01$) and diastolic blood pressure (DBP) ($r = 0.24$; $p = 0.05$). Linear regression analysis revealed the influence of blood pressure on carotid IMT ($\beta = 0.006$; $p = 0.002$, 95% CI: 0.002–0.009). Furthermore, this correlation remained significant in a multivariable linear regression analysis after adjustment for age, some anthropometric and biochemical parameters, and blood pressure as the independent variables (Table 3).

4 Discussion

The number of overweight and obese children and adolescents is currently growing worldwide. Obesity-related metabolic and hemodynamic disturbances such as dyslipidemia, insulin resistance, type 2 diabetes, and hypertension play a fundamental role in the pathogenesis of atherosclerosis. Atherosclerosis can begin in the pediatric population at all ages. Early accumulation of fatty streaks of lipid-engorged macrophages in the intima of an artery has no clinical symptoms but can be accessed *via* B-mode ultrasonography imaging of the thickness of intima-media space of carotid artery wall. Measurement of carotid IMT is an established non-invasive surrogate indicator for the evaluation of subclinical atherosclerosis (Hong 2010; Urbina et al. 2009). Some recent studies have confirmed that exposure to excess adiposity and some cardio-metabolic complications associated with overweight and obesity lead to increased thickness of carotid artery wall in children and adolescents (Reinehr and Wunsch 2011; Fang et al. 2010; Giannini et al. 2008; Iannuzzi et al. 2004).

In the present study we evaluated carotid IMT in several subgroups formed according to the following criteria: obese children under 10 years of age, obese children aged 10 years and older divided according to the presence of MetS criteria of IDF, and the control group of

non-obese peers. We showed that obese children who fulfilled MetS criteria had the highest mean value of IMT, which differed significantly compared to obese children without MetS. Similar findings were obtained by Elshorbagy et al. (2016) based on a study of 60 obese children aged 8–16 years. In that study, significant differences of carotid IMT have also been reported between obese children and the control group, which we did not see in the present study. In another study, Vijayasarathi and Goldberg (2014) have demonstrated that children with metabolic syndrome had a higher mean value of IMT compared to patients with heterozygous familiar hypercholesterolemia and in both of these groups carotid IMT values were higher compared to non-obese patients. Also Reinehr et al. (2008), who studied a group of 264 Caucasian overweight children aged 7–16 years, have found significant correlations between carotid IMT values and metabolic syndrome defined by different MetS criteria established by Viner et al. (2005) and Weiss et al. (2004), but not those established by de Ferranti et al. (2004) and Cook et al. (1988). We did not find significant differences between the whole group of obese children and the control group, probably due to mismatched age of studied children; the difference in the mean age between obese children and the control group was 1.6 years. When we took into account the group of older obese children, whose mean age was similar to the mean age of the corresponding control group, the differences of mean carotid IMT between these groups became greater and statistically significant in relation to children with MetS. This confirms that structural endothelial changes appear to be dependent on coexisting vascular risk factors and their severity (Reinehr and Wunsch 2011).

The main abnormalities in MetS etiology are central obesity and insulin resistance. Giannini et al. (2008), who studied a group of 53 prepubertal obese children, have found correlations between IMT and insulin resistance indices (HOMA, WBISI – whole body insulin sensitivity index, glucose–insulin ratio). Elshorbagy et al. (2016) have shown that epicardial fat mass – an indirect marker reflecting visceral fat – is an independent predictor of carotid IMT in obese children with MetS. In the present study we did not find a link between carotid IMT and surrogate indices of body fat centralization and insulin resistance, nor did we find any differences regarding these parameters between groups of children with and without MetS diagnosis. Systolic blood pressure seems to be an important risk factor of early arterial structural changes in the present study. A linear correlation between carotid IMT and systolic hypertension has been demonstrated by Stabouli et al. (2012) in a study of 53 obese and 78 non-obese patients. In a study by Reinehr et al. (2006), including 96 obese children (mean age 11 years) carotid IMT values have been associated with hypertension and also with impaired glucose metabolism and inflammation. Another study (Aggoun et al. 2008) has shown that elevated blood pressure impairs endothelium function. Furthermore, Weberruß et al. (2016) have explained that non-significant differences in the value of carotid IMT SDS between obese patients and healthy patients could be due to the lack of additional cardiovascular risk factors, like hypertension accompanying obesity. We hypothesize that the lack of significant correlations between all obese children and the control group in our study is due to the small number of children with hypertension. Most of the children with elevated blood pressure belonged to the group with MetS diagnosis, which had the highest mean value of IMT.

Moreover, in this study we observed that older children had slightly higher mean values of IMT than younger ones, despite having a lower BMI SDS. Park et al. (2015) in their systemic review including 7366 children and adolescents have shown an association between IMT and adiposity in children with the mean age $\geq$ 12 years, but not in pre-adolescents. Although we did not find such a relationship, we found that duration of obesity is an important risk factor for endothelial alterations. Also, Reis et al. (2013) have shown in follow-up examinations that duration of overall and abdominal obesity is associated with coronary artery calcification and its progression, independent of the degree of adiposity. It

cannot be ruled out that the higher values of IMT in older children are due to an adaptive response to sheer stress and tensile stress related to higher blood pressure. Probably pressure overload and the co-existence of metabolic risk factors, which are both characteristic of children with MetS, lead to pathological remodeling of artery wall and increased IMT.

In the literature there are only a few reports denying the existence of significantly higher values of carotid intima media wall thickness in obese pediatric population (Aggoun et al. 2008; Tounian et al. 2001). Despite the absence of differences in IMT between obese children and their normal-weight peers, Weberruβ et al. (2016) have shown that obese girls have significantly higher arterial stiffness parameters, which correlate with BMI. Early manifestations of atherosclerosis, such as alterations of endothelial and smooth muscle function, have also been demonstrated in 48 children with severe obesity (BMI z-score 4.4) in a study by Tounian et al. (2001) and in 48 prepubertal obese children in a study by Aggoun et al. (2008). These changes may precede remodeling process of the artery wall, yet they are not advanced enough to result in increased IMT. Presumably, changes in the morphology of carotid artery wall and significantly increased IMT could have appeared in the children from our study group with a longer time of followup. The Bogalusa Heart Study showed that childhood obesity contributes to elevated IMT in adults, independent of adult BMI (Freedman et al. 2008).

Silva et al. (2012), in their meta-analysis covering 16 articles published from 2005 to 2009 comparing IMT between obese and non-obese children, stress the importance of methodology of the IMT measurement. In 12 studies in which the differences in IMT were significant the value of IMT in obese children ranged widely from 0.37 to 0.74 mm. According to these authors, the inconsistency in the results may be explained by the fact that different segments were measured and the number of measurements varied. For example, Reinehr et al. (2006) have measured four values on each side near the bifurcation of the common carotid and have used maximum values for statistical calculations.

An increasing number of clinical studies emphasize the role of obesity in children and adolescents in the development of atherosclerosis in early childhood. Metabolic syndrome, defined as a cluster of interrelated risk factors that promote and enhance the risk of cardiovascular disease development, constitutes a particular risk for early remodeling of artery wall. As the obesity rate increases, the prevalence of metabolic syndrome grows in pediatric population and affects 16–44% of obese children (Friend et al. 2012). In the present study, MetS was diagnosed in 22.2% of patients. It is a global problem, because obesity with its concomitant cardiovascular disorder, and especially metabolic syndrome, can persist into adulthood and promote further growth of carotid IMT (Koskinen et al. 2009). Taking care of children with metabolic syndrome should be one of the methods of prevention of cardiovascular diseases in adulthood.

5 Conclusions

Abdominal obesity and the accompanying components of metabolic syndrome increases carotid intima-media thickness. High systolic blood pressure plays a major role in changing the carotid intima-media complex thickness. Early recognition and primary prevention in children with hypertension and other metabolic cardiovascular risk factors protect against the development of atherosclerosis and thus decrease the incidence of cardiovascular disease in adults. The measurement of IMT may be an additional tool to improve risk stratification of atherosclerosis in children and adolescents with obesity.

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

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 33: 73–80
DOI 10.1007/5584_2017_32
© Springer International Publishing Switzerland 2017
Published online: 13 April 2017

Pro-inflammatory Cytokines in Psychiatric Disorders in Children and Adolescents: A Review

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Abstract

Cytokines are a large group of small proteins which play a significant role in cell signaling and regulate a variety of processes in organisms, including proliferation and differentiation of many cells, mediation in defense reactions and regulation of hematopoiesis. Cytokines can be divided into those with pro- and those with anti-inflammatory properties. In the group of pro-inflammatory cytokines the most important are: IL-1 beta, IL-6, TNF-alpha, and IFN-gamma. Pro-inflammatory cytokines might be involved in the pathophysiology of many psychiatric conditions in adults, but their role in children and adolescents is less clear. The aim of this article is to demonstrate the patterns of pro-inflammatory cytokines in children and adolescents.

Keywords

Adolescents • Anorexia nervosa • Bipolar disorder • Children • Cytokines • Depression • Inflammation • Posttraumatic stress disorder • Psychosis

1 Introduction

Cytokines belong to non-antigen specific, soluble factors. They comprise a large and heterogeneous group of proteins which regulate a variety of immunological processes in the body, primarily inducing the proliferation, differentiation, and chemotaxis of cells by adjusting the time and intensity of the immune response, and then controlling the action and maturation of immune cells. In general, they have a paracrine action, but when excessively activated by

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inflammatory agents, they are secreted into the blood and exert systemic endocrine action. The main feature of cytokines is the pleiotropy, manifested in the fact that one cytokine may have different activities and act on different cell types, and redundancy, manifested in the ability of different cytokines to exhibit the same effect on specific cells. Cytokines exert their effects through the receptors present on the target cells. Usually cytokine receptors consist of extracellular and intracellular domains. Receptors for various cytokines are quite diverse structurally but almost all belong to one of five families of receptor proteins. Binding with a proper receptors leads to the activation of cell signaling pathways, such as the guanosine triphosphate (GTP)ase pathway, members of the mitogen activated protein kinases, Janus kinases (JAKs), the signal transducer and activator of transcription (STAT) protein family, tyrosine-protein kinase Tec, and the proto-oncogene tyrosine-protein kinase Src. Cytokines can be divided into those with pro-inflammatory and those with anti-inflammatory activity. The first group includes interleukin (IL)-1 beta, IL-6, interferon (IF)-gamma, and tumor necrosis factor (TNF)-alpha and in this review we focus primarily on this group of cytokines (Nájera-Medina et al. 2014).

The IL-1 family consists of many cytokines with similar structure (e.g., IL-1 alpha, IL-1 beta, IL-18, and IL-33). These cytokines are produced in various cells from inactive precursors, except IL-1 alpha that itself is a precursor, and converted into active forms by enzymes such as the IL-1 beta converting enzyme. The most important cytokines of the IL-1 group are IL-1 alpha, IL-1 beta, and IL-18. IL-1 beta is responsible for most of the effects induced by IL-1. IL-1 beta is produced mostly by monocytes and macrophages, but also by keratinocytes, chondrocytes, dendritic cells, and many others in response to lipopolysaccharides (LPS), other cytokines, or complement system. IL-1 beta is one of the major regulators of immune and inflammatory response. Its features include the following:

- induction of IL-2 and its receptor production, mainly in lymphocytes recognizing the antigen;
- stimulation of basophils to release histamine;
- stimulation of IFN-gamma synthesis by T lymphocytes and that of IL-6 by macrophages, endothelial cells, and fibroblasts;
- stimulation of B cells proliferation and differentiation;
- increase the number of neutrophils and monocytes by the colony stimulating growth factors (CSF): granulocyte (G-CSF), macrophage (M-CSF), and granulocyte/macrophage CSF (GM-CSF);
- increase the number of osteoclasts;
- stimulation of fibroblasts and osteoclasts to produce collagenases and prostaglandins (Sims and Smith 2010).

IL-2 exerts its effects on many different cell types especially T lymphocyte. One of the most rapid consequences of T cell activation through its receptors is *de novo* synthesis of IL-2. This is followed by the expression of a high affinity IL-2 receptor. Major biological activities of IL-2 include the following:

- CD4: induction of antigen-specific clones expansion *via* proliferative and anti-apoptotic mechanisms, stimulation of other cytokines production, induction of activated T cells apoptosis *via* Fas ligand, a type II transmembrane protein belonging to the tumor necrosis factor family, signaling, and induction of CD4+CD25+ T regulatory cells development;
- CD8: induction of antigen-specific clones expansion, amplification of cytokine secretion, and cytotoxic activity;
- B cells: stimulation of proliferation and antibody secretion;

- natural killer (NK) cells: enhancement of proliferation, cytotoxic action and cytokine production. IL-2 is produced mainly by Th1 and Tc lymphocytes after the interaction with antigen. In fact, the majority of IL-2 is derived from activated CD4+ T cells (Gaffena and Liu 2004).

IL-6 is the most important member of the family of cytokines, which present similar effects. Members of the interleukin-6 (IL-6) family include IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary inhibitory factor (CNTF), cardiotropin-1 (CT-1), cardiotrophin-like related cytokine, and stimulating neurotrophin-1/B-cell stimulating factor 3 (NNT-1), neuropoietin (NPN), IL-27, and IL-31. IL-6 is a pleiotropic cytokine that is a major factor in regulating defense mechanisms in the organism by adjusting the response against pathogens, acute phase reaction and hematopoiesis. IL-6 is produced mainly by monocytes and macrophages induced by IL-1, interferons, and TNF-alpha, LPS, and viruses. It acts primarily on B cells that, under its influence, differentiate into cells producing immunoglobulins of different classes. IL-6 participates in the activation of antigens recognizing T lymphocytes. It also induces the growth of keratinocytes, neuronal differentiation, and secretion of vascular endothelial growth factor (VEGF). IL-6 is a major stimulator of acute phase protein production. It has pyrogenic effects, which together with TNF-alpha and interferons elevates body temperature and stimulate the production of prostaglandin (Scheller et al. 2011).

TNF-alpha belongs to the superfamily of molecules of the tumor necrosis factors, which includes at least 22 molecules of similar structure. TNF-alpha is produced primarily by monocytes and macrophages, especially following the LPS stimulus. TNF-alpha is one of the main acute phase reaction cytokines. It can also enhance the proliferation of B and T lymphocytes and differentiation of B lymphocytes. TNF-alpha is part of the response against pathogens, increases the cytotoxicity of monocytes and macrophages, and activates neutrophils by acceleration of their release from the bone marrow. The next important role is inducing the expression of histocompatibility antigens I and II, together with IFN-gamma. TNF-alpha is a pro-inflammatory cytokine that exerts both homeostatic and pathophysiological roles in the central nervous system. In the healthy CNS, TNF-alpha has regulatory functions on crucial physiological processes such as synaptic plasticity (Kaneko et al. 2008), learning and memory, sleep (Krueger 2008), and food intake (Plata-Salamán 2001).

Interferons form a cytokine group involved in the response to infection. Human cells produce interferons type I, II, and III. Interferon type II or IFN-gamma is produced mainly by natural killer (NK) and natural killer T (NKT) cells as part of the innate immune response, and by Th1 CD4 and CD8 cytotoxic T lymphocyte (CTL) effector T cells once the antigen-specific immunity develops (Schoenborn and Wilson 2007). IFN-gamma causes inhibition of the proliferation of virus-infected cells and activation of the defense mechanisms of the immune system. It induces the maturation of different cells and increases the expression of histocompatibility antigens I and II (MHC I, MHC II) in different target cells.

For many years, the relationship between inflammation, including the activity of pro-inflammatory cytokines, and psychiatric disorders has been considered, with most research focusing on the adult population. However, there are increasing numbers of reports about the activity of pro-inflammatory cytokines in psychiatric disorders in children and adolescents. The populations of adults and children differ in terms of cytokine activity, so it is not always possible to translate results from studies on adults to pediatric patients.

2 Major Depressive Disorder (MDD)/Dysthymia/Suicidality

The first report presenting the association between depression and pro-inflammatory

cytokines was delivered by Smith (1991), in which the author linked increased secretion of cytokines by macrophages with depressive symptoms. Through the years, this relation has been well studied by many researchers all over the world. Mills et al. (2013), in an excellent review article, have described the effects of cytokines on the following pathways in the human brain: 1) neurotransmitters - reduction in the level of serotonin by mediating the catabolism of tryptophan into kynurenine; 2) hypothalamic–pituitary–adrenal (HPA) axis - increasing the level of corticotrophin-releasing hormone (CRH) and disturbing the function of the glucocorticoid receptor; and 3) neurogenesis - changing the cell proliferation in the hippocampus. All such effects have a potential to contribute to the development of depression. The article has since spurred clinical research on the role of pro-inflammatory cytokines in depression in children and adolescents. A great majority of studies have been devoted to plasma/serum levels of IL-6 and TNF-alpha, and also of IL-1 beta, IL-2, and INF-gamma. The results are not conclusive in terms of the concentration of IL-1 beta in children and adolescents in depression or dysthymia. IL-1 beta has been found to be elevated in dysthymic (Brambilla et al. 2004) and depressed untreated patients compared to control subjects (Henje-Blom et al. 2012), but unchanged in major depressive disorder (MDD) patients (Gabbay et al. 2009a; Brambilla et al. 2004). High levels of IL-1 beta can be associated with poor response to treatment with serotonin-specific reuptake inhibitors (SSRIs) in adolescents with MDD and anxiety disorders, and fluoxetine does not reduce the IL-1 level (Amitai et al. 2016).

Both IL-2 and IL-6 are significantly elevated in depression in children and adolescents in some reports (Pallavi et al. 2015; Henje-Blom et al. 2012) in both non-medicated and medicated groups in comparison to controls. The issue is, however, contentious, as others have found no major alterations in the level of IL-6 (Hood et al. 2012; Gabbay et al. 2009b). Studies on adolescents also have yielded contradictory results regarding the effect of SSRIs on the IL-6 level. Henje-Blom et al. (2012) have postulated that the level of IL-6 is associated with SSRI treatment and is elevated in patients with untreated depression, but reduced in medicated patients. A study by Amitai et al. (2016) has indicated that, similar to IL-1 beta, the level of IL-6 is not reduced by fluoxetine and high levels of this cytokine are associated with poor response to treatment with SSRIs. Likewise, studies on TNF-alpha in adolescent depression have found either its decreased level in dysthymic and MDD patients or inappreciable changes compared to controls (Henje-Blom et al. 2012; Brambilla et al. 2004; Gabbay et al. 2009a, b). Antidepressant treatment significantly reduces TNF-alpha level (Amitai et al. 2016). There are two reports that show a significantly elevated level of IFN-gamma in depressed adolescents in comparison to controls (Gabbay et al. 2009a, b). Henje-Blom et al. (2012), however, have failed to confirm an increase in IFN-gamma in adolescents with MDD.

Studies concerning differences in cytokine patterns in adolescent depression with suicidal symptomatology have found no differences in the levels of IL-6 and IL-1 beta and a decrease in TNF-alpha in suicidal adolescents with MDD compared to non-suicidal adolescents. In contrast, IFN-gamma is elevated in suicidal and non-suicidal groups of adolescents with MDD (Gabbay et al. 2009b). The limitations of that study were small groups of patients (30 persons and 15 healthy controls) and the fact that half of the patients received psychotropic medications. Pandey et al. (2012) have examined teenage (12–20 years old) 'suicide victims' brains and show the increased mRNA levels of TNF-alpha, IL-1 beta, and IL-6 and the protein levels of TNF-alpha and IL-1 beta in the Brodmann area 10 of the prefrontal cortex.

The need for further studies on pro-inflammatory cytokines in childhood depression is demonstrated by the fact that high levels of IL-6 can lead to the occurrence of depressive symptoms. In a study by Miller and Cole (2012), high levels of IL-6 are strongly associated with the development of depression six months later in those with a history of childhood adversity.

One population-based longitudinal study of 4,500 individuals has revealed that children with a higher level of IL-6 at age 9 are at greater risk of developing depressive disorders at age 18 (Khandaker et al. 2014).

3 Bipolar Disorder

There is a lack of understanding about the presence of pro-inflammatory cytokines in pediatric-onset bipolar disorder. To our knowledge, there is only one study on pro-inflammatory cytokines concerning children and teenagers with bipolar disorder. Miklowitz et al. (2016) have compared a small group of adolescents with bipolar disorder with depressive patients and with healthy subjects and demonstrates that adolescents with bipolar disorder had a significantly higher plasma level of IL-1 beta than healthy controls.

Goldstein et al. (2015) have tested a mixed group of 123 adolescents and young adults (age range between 13.4–28.3 years) with bipolar disorder for the serum levels of IL-6 and TNF-alpha. They demonstrate positive correlations between the IL-6 level and disease duration, and the percentage of weeks with mood symptoms and the percentage of weeks with suicidal ideation. An inverse correlation has been found between the percentage of weeks with euthymia and IL-6 level. The maximum severity of depression and mania was associated with IL-6 level. The level of TNF-alpha was positively correlated with the percentage of weeks with psychosis. Surprisingly, treatment with psychotropic medication, antidepressants, stimulants, and psychosocial treatment was associated with higher concentrations of IL-6.

4 Schizophrenia

The association between pro-inflammatory cytokines and psychosis has been well known since Smith (1992) proposed the theory of deficient macrophages that are unable to control the secretion of IL-2 from T lymphocytes. This theory has arisen from the observation that in healthy volunteers an increase of IL-2 leads to regular symptoms of schizophrenia. In the last twenty years, many authors have studied the connection between pro-inflammatory factors and psychosis, but mainly in adult groups.

Falcone et al. (2015) have reported that children with psychosis have elevated rates of TNF-alpha, IL-1 beta, IL-6, and IFN-gamma in comparison with control groups consisting of inpatients with other psychiatric diseases. The problem, according to Khandaker and Dantzer (2016), is that it is hard to determine whether the cytokine elevation is a cause or result of psychosis. One population-based longitudinal study of 4,500 individuals revealed that 9 years old children with elevated levels of IL-6 have two-fold increased risk of developing the psychotic disorder at age of 18 (Khandaker et al. 2014). Further studies are required to confirm cytokine patterns in psychosis in children and to distinguish any causal link between psychosis and elevated levels of pro-inflammatory cytokines.

5 Anorexia Nervosa

Although obesity is strongly associated with inflammation, increased levels of pro-inflammatory cytokines have been found in eating disorders with weight loss, such as anorexia nervosa. Solmi et al. (2015) have summed up the possible reason for this finding, noting the fact that IL-6 and IL-1 beta are involved in the control of body weight by regulation of the expression of hypothalamic neuropeptides (Señarís et al. 2011). Further, TNF-alpha mediates the production of anorexigenic peptides (Inui 2001).

There is a lack of evidence for the claim that higher levels of pro-inflammatory cytokines occur in eating disorders in adolescents. In a longitudinal study including 12 pediatric subjects with anorexia nervosa, Shimizu et al. (2005) have found an elevated level of TNF-alpha in comparison to control subjects. The level of IL-6 is also higher in patients than controls in a study by Misra et al. (2006), but no correlation

has been found between IL-6 and increases in body mass or in duration of illness. In a subgroup of girls aged 13–17 years, Ostrowska et al. (2015) have found increased levels of IL-1 beta, IL-6, and TNF-alpha and point to their possible influence on bone markers. Those authors postulate that elevated pro-inflammatory cytokines could factor in the development of osteoporosis in subjects with anorexia nervosa. Nova et al. (2002) have examined the *in vitro* production of IFN-gamma, IL-2, TNF-alpha, IL-6, and IL-1 beta from stimulated peripheral blood monocytes obtained from 44 teenage subjects with anorexia nervosa. The authors demonstrate a reduction in TNF-alpha and IL-6, and an increase in IL-1 in the group, both at admission and one month after discharge from the hospital. No differences in the level of IL-2 and a decrease in IFN-gamma have been found on patients' discharge from the hospital. Thus, available evidence suggests that anorexia nervosa rather than obesity is associated with inflammation, as assessed from the content of pro-inflammatory cytokines.

6 Anxiety Disorders

There is virtually one study that has directly examined children and adolescents for cytokines levels in anxiety disorders. In the study, Pervanidou et al. (2007) have measured the serum concentrations of IL-6 in children who experienced motor vehicle accidents. The authors have found that IL-6 is increased in those who developed post-traumatic stress disorder, six months later.

Mitchell and Goldstein (2014) have reviewed studies on pro-inflammatory cytokines in both pediatric and adult populations and found several reports that demonstrate the presence of post-traumatic elevation of IL-6 (Baker et al. 2001; Maes et al. 1999). The elevation tends to normalize in children, but remains prolonged in adults. The prolongation has to do with the influence of the hypothalamic pituitary axis (HPA), being chronically activated in co-morbidities in adults, on IL-6.

The literature on the link between pro-inflammatory cytokines and inflammation in psychiatric disorders in the pediatric population rarely refers to the concept of anti-inflammatory treatment in addition to psychotropic medication in psychiatric diseases. To our knowledge, there is only one study on this subject that includes adolescents. Amminger et al. (2010) have addressed the use of anti-inflammatory treatment with omega-3 polyunsaturated fatty acids (PUFAs) in adolescent psychoses in a randomized, double-blind, placebo-controlled trial. The authors demonstrate that omega-3 PUFAs significantly reduced positive, negative, and general symptoms of psychoses in comparison with placebo.

In contrast, the adult literature abounds in similar reports, in both affective disorders and psychoses. Anti-inflammatory drugs combined with antipsychotics may have a positive influence on amelioration of psychotic (Chaudhry et al. 2013; Laan et al. 2010; Müller et al. 2002) and depressive symptoms (Husain et al. 2015; Gałecki et al. 2009; Mendlewicz et al. 2006). These findings need confirmation in the pediatric population, because it would seem to be a promising idea to treat the first episode of a psychiatric disorder in children with adjunctive anti-inflammatory therapy in order to counteract the possibility of remitting morbidity in later on, which could adversely affect quality of adult life.

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

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 33: 81–92
DOI 10.1007/5584_2017_22
© Springer International Publishing Switzerland 2017
Published online: 13 April 2017

Renalase in Children with Glomerular Kidney Diseases

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Abstract

Studies suggest that renalase, a renal catecholamine-inactivating enzyme, plays a major role in the pathogenesis of kidney and cardiovascular diseases in adults. This study seeks to determine the role of renalase in children with glomerular kidney diseases. We evaluated the serum renalase, arterial stiffness, intima-media thickness, blood pressure, and clinical and biochemical parameters in 78 children (11.9 ± 4.6 years of age) with glomerulopathies such as idiopathic nephrotic syndrome (40 cases), IgA nephropathy (12 cases), Henoch-Schönlein nephropathy (12 cases), and other glomerulopathies (14 cases). The control group consisted of 38 healthy children aged 11.8 ± 3.3 years. The mean renalase was 25.74 ± 8.94 μg/mL in the glomerulopathy group, which was not significantly different from the 27.22 ± 5.15 in the control group. The renalase level did not differ among various glomerulopathies either. However, proteinuric patients had a higher renalase level than those without proteinuria (28.43 ± 11.71 *vs.* 24.05 ± 6.23, respectively; $p = 0.03$). In proteinuric patients, renalase correlated with daily proteinuria. In the entire glomerulopathy group, renalase correlated with age, systolic central blood pressure (BP), diastolic peripheral and central BP, mean peripheral and central BP; peripheral diastolic BP Z-score, glomerular filtration rate, cholesterol, triglycerides, and pulse wave velocity. We conclude that in children with glomerulopathies renalase, although

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basically not enhanced, may underlie blood pressure elevation and arterial damage.

Keywords
Arterial stiffness • Blood pressure • Children • Glomerular kidney diseases • Intima-media thickness • Proteinuria • Renalase • Sympathetic system

1 Introduction

Glomerulopathies are immune diseases manifesting as asymptomatic proteinuria or erythrocyturia, and nephrotic or nephritic syndrome. Glomerular kidney diseases are a cause of end-stage renal disease in children in 24% of cases in Poland (Żurowska et al. 2006) and 5–29% of cases worldwide (Chesnaye et al. 2014; Harambat et al. 2012). In recent years, increases in cardiovascular morbidity due to disturbed lipid metabolism and coagulation and in sympathetic activation were found in adult patients with glomerulopathies (Patel 2010). Pharmacotherapy of glomerulopathies involves the use of immunosuppressive and renoprotective agents. Immunosuppressive regimens are based on high doses of glucocorticoids, commonly together with other immunosuppressive medications, e.g., cyclosporine A, azathioprine, mycophenolate mofetil, or alkylating cytostatics. Glucocorticoids may predispose to arterial hypertension, central obesity, and hyperlipidemia, all of which pose a risk for adverse changes in the cardiovascular system regardless of the underlying disease (Nakamura et al. 2010).

Increased activation of the sympathetic nervous system has been found in chronic kidney diseases (Vink et al. 2013; Schlaich 2009). Elevated urinary catecholamine excretion is present in adults with glomerulonephritis and normal renal function (Rahman et al. 1993; Ishii et al. 1983). Nephrotic syndrome induced in experimental animals is also characterized by increased activity of the sympathetic system (Camici 2007; DiBona et al. 1996; Herman et al. 1989). Moreover, obesity, which is now a major public health problem and a common complication of chronic glucocorticoid treatment, is associated with increased sympathetic and decreased parasympathetic activation (Soares-Miranda et al. 2011), which may be caused by leptin secreted from adipose tissue (Koszowska et al. 2014; Guizar et al. 2005).

The reason for excessive sympathetic activity in patients with glomerular kidney diseases has not been determined. One of the possible links is renalase that plays a vital role in the renal regulation of the sympathetic system in health and disease. Human renalase is produced mainly by renal proximal tubular cells, but its expression also takes place in glomeruli, distant tubules, heart, muscles, vascular endothelium, and liver, as well as in the peripheral and central nervous systems. In humans, renalase gene is placed on chromosome 10 (10q23.33) and consists of 311,000 base pairs encoding seven exones (Xu et al. 2005). Alternative splicing leads to the formation of four different proteins (renalase 1–4). Only is renalase 1 detected in human blood samples, which means that renalase 2–4 probably have different functions than renalase 1 (Hennebry et al. 2010; Desir 2009). Renalase inactivates catecholamines in the presence of the cofactors flavine dinucleotide (FAD) and nicotinoaminoadenine dinucleotide (NAD) catalyzing the process of catecholamine oxidation (Desir and Peixoto 2014). In experimental studies, serum renalase concentration is low in mice with renal failure and exogenous renalase supplementation normalizes blood pressure and reverses myocardial fibrosis, left ventricular hypertrophy, and renal injury (Yin et al. 2016;

Gu et al. 2011). Likewise, knock-out mice without renalase gene have a high level of circulating catecholamines and display a marked sensitivity to myocardial and renal ischemia (Wu et al. 2011). In humans, serum renalase concentration correlates negatively with renal function (Malyszko et al. 2011; Przybylowski et al. 2011), and its highest concentration is found in patients with end-stage renal disease (Zbroch et al. 2012). In observational studies, single nucleotide polymorphisms of the renalase gene has been associated with the development of primary arterial hypertension (Zhao et al. 2007), heart failure (Farzaneh-Far et al. 2010), and stroke (Buraczynska et al. 2011).

The studies evaluating renalase and its relation to cardiovascular risk in children with glomerular kidney diseases are still missing in the literature. Thus, this study seeks to assess the level of renalase in children with glomerular kidney diseases and to find the relation between renalase content and clinical, biochemical, and cardiovascular parameters in this group of patients.

2 Methods

We studied 78 children (47 boys and 31 girls) aged from 4 to 18 years (mean 11.9 ± 4.6 years) treated in a single tertiary pediatric nephrology center for glomerular kidney diseases such as idiopathic nephrotic syndrome in 40 (51.3%), IgA nephropathy in 12 (15.4%), Henoch-Schönlein nephropathy in 12 (15.4%), and other glomerulopathies in 14 cases (17.9%). In the subgroup of children with other glomerulopathies, the following kidney diseases were diagnosed: lupus nephropathy in 4, membranous nephropathy in 2, membrano-proliferative glomerulonephritis in 1, C1q nephropathy in 1, congenital nephrotic syndrome (Pierson's syndrome) in 1, focal and segmental glomerulosclerosis in 1 case, and in 4 patients the background of chronic glomerulonephritis remained undetermined. All patients with idiopathic nephrotic syndrome in whom remission was achieved (36 patients) were examined in the remission phase.

We evaluated the serum renalase content in all children studied using a commercially available ELISA kit (Cloud-Clone, Houston, TX, USA). The assay measures a total concentration of renalase-1 without assessing its activity. For the measurement, peripheral venous blood was drawn after overnight fasting. After centrifugation, serum samples were frozen and stored at −80 °C. In addition, the following demographic and biochemical indices were evaluated in all children: age, gender, presence of arterial hypertension, BMI Z-score, and medications used, total protein (g/dL), albumin (g/dL), total, LDL, and HDL cholesterol (mg/dL), uric acid (mg/dL), and daily urinary protein loss (mg/kg/24 h) using standard laboratory methods. Hypoproteinemia was defined as a total protein level <6.0 g/dL, hypoalbuminemia as an albumin level <3.5 g/dL; hypercholesterolemia as a cholesterol level ≥200 mg/dL, hypertriglyceridemia as a triglyceride level ≥100 mg/dL (children aged 0–9 years) or ≥130 mg/dL (children aged 10–19 years); all in the serum (Expert Panel on Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children and Adolescents: Summary Report 2011). Nephrotic proteinuria was defined as a protein loss >50 mg/kg/24 h. Glomerular filtration rate (mL/min/1.73 m^2) was calculated using the Schwartz formula (Schwartz et al. 2009).

Cardiovascular status was assessed from the level of peripheral blood pressure, carotid intima media thickness by 2D ultrasonography, pulse wave velocity, augmentation index corrected for 75 beats/min (AIx75HR), and central blood pressure. Peripheral blood pressure was measured oscillometrically in each patient on the right arm using the Welch Allyn ASM 300 Patient Monitor device (Welch Allyn Inc., Skaneateles Falls, NY, USA) and expressed in mm Hg and Z-score (Kułaga et al. 2012).

The intima-media thickness (cIMT) was defined as the mean distance from the leading edge of the lumen-intima interface to the leading edge of the media adventitia interface of the far wall, approximately 1 cm proximal to the carotid

Table 1 Clinical characteristics of children with glomerulopathies and healthy control subjects

	Glomerulopathy	Healthy subjects	p
Age (yr)	11.9 ± 4.6	11.8 ± 3.3	ns
Gender (boys/girls)	47/31	19/19	–
Idiopathic nephrotic syndrome (n, %) IgA nephropathy (n, %) Henoch-Schönlein nephropathy (n, %) Other glomerulopathies (n, %)	40 (51.3) 12 (15.4) 12 (15.4) 14 (17.9)	–	–
BMI Z-score	0.78 ± 1.03	–0.19 ± 0.78	**0.004**
Arterial hypertension (n, %)	38 (48.7)	–	–
Prednisone (n, %) Prednisone (mg/kg/24 h)	56 (71.8) 0.44 ± 0.43	–	–
Cyclosporine A (n, %) Mofetil mycophenolate (n, %) Azathioprine (n, %) Tacrolimus (n, %) Cyclophosphamide (n, %)	24 (30.8) 12 (15.4) 7 (9.0) 3 (3.8) 2 (2.6)	–	–
Enalapril (n, %) Amlodipine (n, %) Metoprolol (n, %) Propranolol (n, %) Hydrochlorothiazide (n, %) Spironolactone (n, %)	58 (74.4) 14 (18.0) 3 (3.8) 1 (1.3) 2 (2.6) 1 (1.3)	–	–

BMI body mass index, *ns* non-significant

bulb. Six determinations of cIMT, three on the left and three on the right side, were obtained and averaged (Urbina et al. 2009). The cIMT was measured using Aloka Prosound Alpha 6 ultrasound system and a 13-MHz linear transducer (Hitachi Aloka Medical, Mitaka, Japan).

Peripheral pressure waveforms were recorded from the radial artery at the right wrist, using applanation tonometry. After 20 sequential waveforms had been acquired, a validated generalized transfer function was used to generate the corresponding central aortic pressure waveform. We evaluated the following parameters: heart rate (HR) (beats/min; bpm), aortic systolic (AoSP), diastolic (AoDP), and mean blood pressure (AoMBP) (all in mmHg), and the augmentation index corrected for heart rate of 75 beats/min (AIx75HR) (%). Only high-quality recordings, defined as in-device quality index >80%, were included in the analysis. Pulse wave velocity (PWV) was calculated as a difference in the carotid-to-femoral path length divided by the difference in R wave to the foot of the pressure wave taken from the superimposed ECG and pressure tracings. The distance traveled by the flow wave was measured with an external tape measure over the body surface, as the distance from the right carotid sampling site to the manubrium, subtracted from the distance from the manubrium to the right femoral sampling site. All pulse wave velocity measurements were performed in the sitting position in a quiet, temperature-controlled room (20 ± 5 °C) after a 5-min rest. Arterial pulse waveform and aortal pulse wave velocity were evaluated by the same investigator (PS) using a Sphygmocor device (AtCor Medical Pty Ltd., Sydney, Australia).

The control group consisted of 38 healthy children (19 boys and 19 girls) aged 11.8 ± 3.3 years. In these children we assessed the following parameters: age, gender, BMI Z-score, serum renalase level, peripheral blood pressure, cIMT, PWV, AIx75HR, and central blood pressure using the above-described methods.

Variables were presented as means ±SD. Normality of data distribution was tested using the Shapiro-Wilk test. Differences between mean values were tested using the Student *t*-test and

Table 2 Renalase content and biochemical indices in children with glomerulopathies

Renalase (μg/mL)	25.7 ± 8.9
GFR (mL/min/1.73m^2)	108.1 ± 27.0
Total protein (g/dL)	6.8 ± 0.9
Albumin (g/dL)	4.1 ± 0.7
Total cholesterol (mg/dL)	214.4 ± 85.1
HDL cholesterol (mg/dL)	65.5 ± 22.5
LDL cholesterol (mg/dL)	122.1 ± 70.9
Triglycerides (mg/dL)	129.4 ± 84.4
Uric acid (mg/dL)	5.4 ± 1.4
Proteinuria (n, %) Proteinuria (mg/kg/24 h)	30 (38.5%) 45.3 ± 44.6

GFR glomerular filtration rate, *HDL* high density lipoprotein, *LDL* low density lipoprotein

one-way ANOVA or the Mann-Whitney U and Kruskal-Wallis tests, as appropriate. Correlations between variables were evaluated using Pearson and Spearman's correlation coefficients. Variables related to the renalase level with a p-value <0.05 were analyzed by a multivariate analysis with a stepwise regression. A p-value <0.05 was considered statistically significant. Statistical elaboration was performed using Statistica 12.0 PL software (StatSoft, College Station, TX, USA).

3 Results

The most frequent glomerular disease was idiopathic nephrotic syndrome, present in more than half of the children studied (Table 1). Twenty (25.6%) out of the 78 children were overweight and 12 (15.4%) were obese. Arterial hypertension was found in almost half of the children. Fifty six (71.8%) children were treated at the time of evaluation with prednisone at a dose slightly below 0.5 mg/kg/24 h. As many as 42 (53.8%) children received immunosuppressants of which cyclosporine A was the most frequently used. Fifty eight (74.4%) of the children received enalapril and 14 (18.0%) were treated with more than one antihypertensive drug. The glomerulopathy and control groups did not differ significantly in terms of age and gender. However, children with glomerulopathies had a higher BMI Z-score.

Serum renalase and biochemical data in the children investigated are shown in Table 2. The content of renalase ranged was from 13.4 to 59.3 μg/mL, with the mean of 25.7 ± 8.9 μg/mL. Fifty eight (74.3%) children had GFR ≥ 90 mL/min/1.73m^2, 18 (23.1%) had GFR between 89 and 60 mL/min/1.73m^2, and 2 (2.6%) had GFR below 60 mL/min/1.73m^2. Two girls aged 12.9 and 16.9 years, with severe treatment-resistant Henoch-Schönlein nephropathy, arterial hypertension, and proteinuria of 79.5 and 14.3 mg/kg/24 h were in stage III of chronic kidney disease with GFR 41.6 and 57.5 mL/min/1.73m^2, respectively. Hypoproteinemia was present in 9 (11.5%) and hypoalbuminemia in 8 (10.3%) patients. Hypercholesterolemia was present in 27 (34.6%) and hypertriglyceridemia in 24 (30.8%) patients. At the time of evaluation, proteinuria was found in 30 (38.5%) out of the 78 children, with 7 (9.0%) having nephrotic range proteinuria.

Serum renalase content, blood pressure, and cardiovascular variables in children with glomerulopathies and in the control group are shown in Table 3. We found comparable levels of renalase in both groups; 25.7 ± 8.9 *vs.* 27.2 ± 5.2, respectively. Heart rate, and both peripheral and central blood pressure were all significantly higher in children with glomerulopathies than in healthy subjects. Also, key vascular variables such as carotid intima media thickness and pulse wave velocity were significantly higher in children with glomerulopathies. In addition, augmentation index was more positive in these children, but the difference did not reach statistical significance.

Serum renalase content in children with glomerulopathies and in healthy children is presented in Fig. 1. There were no significant differences between the level of renalase in both groups. Moreover, we found no significant differences in renalase level among children with different glomerulopathies or between boys and girls with glomerular kidney diseases. In contrast, proteinuric patients were characterized by a significantly higher renalase level compared to those without proteinuria at the time of

Table 3 Central and peripheral blood pressure and vascular status in children with glomerulopathies and in the control group

Variable	Glomerulopathies	Controls	p
Renalase (μg/mL)	25.7 ± 8.9	27.2 ± 5.2	ns
HR (beats/min)	88.8 ± 17.3	79.9 ± 12.5	0.01
SBP (mmHg)	118.0 ± 11.3	108.2 ± 7.9	<0.0001
SBP Z-score	0.93 ± 1.12	0.10 ± 0.68	0.0003
DBP (mmHg)	72.0 ± 12.0	63.7 ± 7.2	0.0007
DBP Z-score	0.81 ± 1.04	0.04 ± 0.67	0.0004
AoSBP (mmHg)	100.4 ± 11.8	91.1 ± 6.6	0.0001
AoDBP(mmHg)	73.6 ± 12.0	65.2 ± 7.5	0.0007
AoMBP (mmHg)	86.9 ± 11.6	78.7 ± 7.0	0.0005
cIMT (mm)	0.45 ± 0.06	0.42 ± 0.04	0.003
PWV (m/s)	5.0 ± 0.8	4.3 ± 0.6	<0.0001
AIx75HR (%)	5.2 ± 17.5	3.5 ± 11.7	ns

HR heart rate, *SBP* peripheral systolic blood pressure, *DBP* peripheral diastolic blood pressure, *AoSBP* aortic systolic blood pressure, *AoDBP* aortic diastolic blood pressure, *AoMBP* aortic mean blood pressure, *cIMT* carotid intima-media thickness, *PWV* pulse wave velocity, *AIx75HR* augmentation index corrected for heart rate 75 beats/min, *ns* non-significant

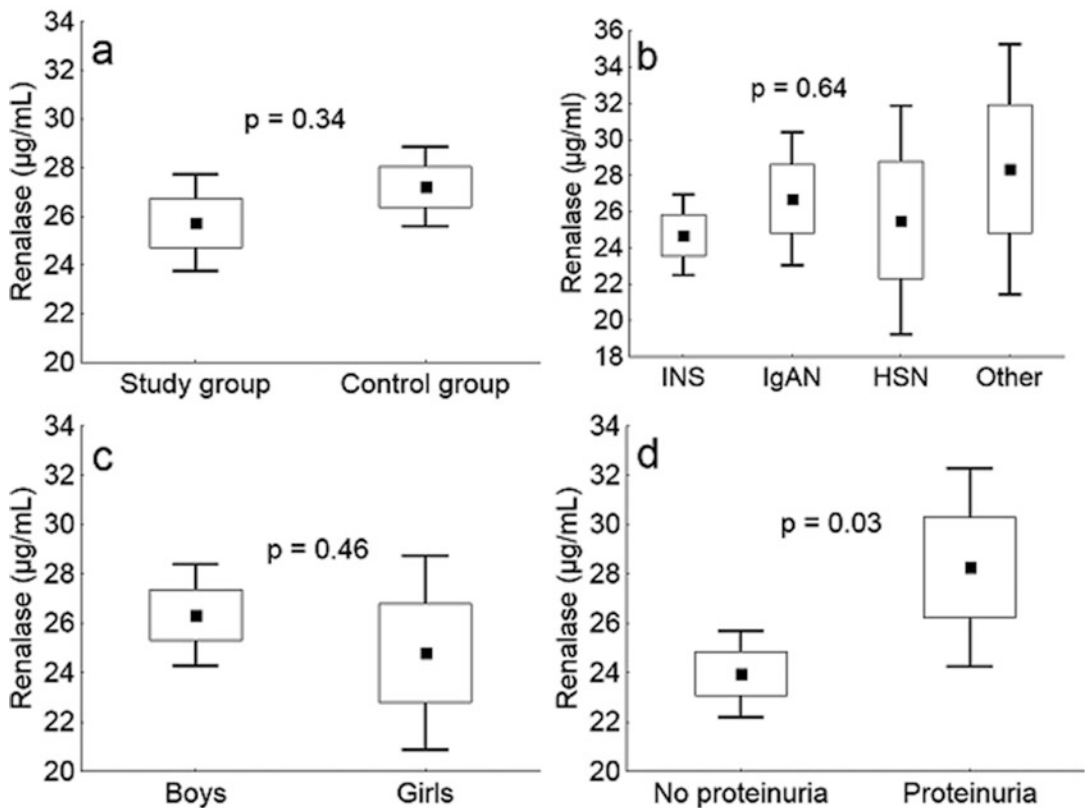

Fig. 1 (**a**) serum renalase in children with glomerulopathies and in healthy children; (**b**) serum renalase in children with idiopathic nephrotic syndrome (INS), IgA nephropathy (IgAN), Henoch-Schönlein nephropathy (HSN), and other glomerulopathies; (**c**) serum renalase in boys and girls with glomerulopathies; and (**d**) serum renalase in children with glomerulopathies with and without proteinuria. *Middle points* in graphs represent mean values, *boxes* represent SE deviation, and whisker are 1.96SE

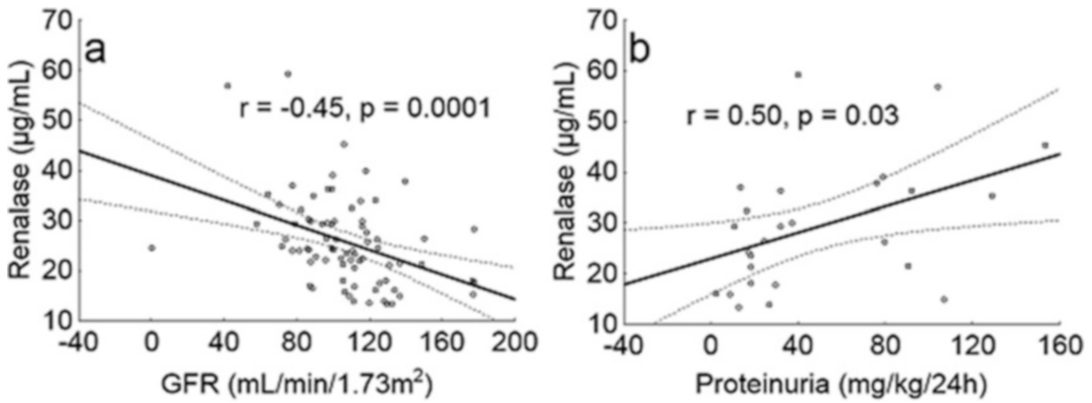

Fig. 2 Serum renalase in children with glomerulopathies: (**a**) relationship between renalase and glomerular filtration rate (GFR) and (**b**) relationship between renalase and proteinuria in 30 proteinuric children with glomerulopathies. *Solid lines* are linear regression *lines* and *dotted* lines represent 95% confidence intervals

Table 4 Analysis of factors affecting renalase level in children with glomerular kidney diseases by stepwise multiple regression analysis

Variable	β	p
Age (years)	–0.02	ns
GFR (mL/min/1.73 m^2)	–0.40	0.02
Proteinuria (mg/kg/24 h)	0.45	0.01
Total cholesterol (mg/dL)	0.40	0.02
Triglycerides (mg/dl)	0.45	0.01
DBP (mmHg)	0.42	0.02
DBP (Z-score)	0.43	0.01
MBP (mmHg)	0.41	0.02
AoSBP (mmHg)	0.37	0.04
AoDBP (mmHg)	0.41	0.02
AoMBP (mmHg)	0.41	0.02
PWV (m/s)	0.25	ns

GFR glomerular filtration rate, *DBP* peripheral diastolic blood pressure, *MBP* mean peripheral blood pressure, *AoSBP* aortic systolic blood pressure, *AoDBP* aortic diastolic blood pressure, *AoMBP* aortic mean blood pressure, *PWV* pulse wave velocity, *ns* non-significant

evaluation; 28.4 ± 11.7 *vs.* 24.1 ± 6.2 μg/dL, p = 0.03. Four girls with lupus nephritis of the mean age of 16.8 ± 2.1 years had a distinctly higher serum renalase of 38.5 ± 14.8 μg/dL. The four girls had stage II chronic kidney disease with the mean GFR of 78.3 ± 11.3 mL/min/ 1.73m^2, two of them had accompanying subnephrotic proteinuria, and another two had arterial hypertension.

Relationships between serum renalase and clinical and biochemical parameters in children with glomerulopathies are presented in Fig. 2. Renelase level was inversely associated with GFR ($r = -0.45$; p = 0.0001;) and positively associated with daily urinary protein loss in 30 proteinuric patients ($r = 0.50$; p = 0.03). In addition, renalase was associated with children's age ($r = 0.25$; p = 0.04), total cholesterol ($r = 0.27$; p = 0.04), and triglycerides ($r = 0.30$; p = 0.02;). There also was a trend toward an association between serum renalase and uric acid in a subgroup of 40 patients with idiopathic nephrotic syndrome (r = 0.31; p = 0.05).

In 78 children with glomerulopathies we found associations between serum renalase and, on the other side, peripheral diastolic blood pressure expressed in mmHg (r = 0.37; p = 0.01) and as Z-score (r = 0.34; p = 0.02), mean blood pressure expressed in mmHg ($r = 0.36$; p = 0.01), and all central blood pressure indices: aortic systolic ($r = 0.32$; p = 0.03), aortic diastolic ($r = 0.37$; p = 0.01), and aortic mean blood pressure ($r = 0.36$; p = 0.01). In addition, there was a trend toward an association between renalase and aortic pulse wave velocity ($r = 0.25$; p = 0.07). There was no apparent

Table 5 Correlation analysis of renalase level and clinical and vascular parameters in healthy children

Variables	*r*	p
Age (years)	–0.37	0.02
GFR (mL/min/1.73m^2)	0.04	ns
HR (beats/min)	–0.05	ns
SBP (mmHg)	0.12	ns
SBP Z-score	0.25	ns
DBP (mmHg)	0.16	ns
DBP Z-score	0.24	ns
AoSBP (mmHg)	0.17	ns
AoDBP (mmHg)	0.17	ns
AoMBP (mmHg)	0.23	ns
cIMT (mm)	0.002	ns
PWV (m/s)	–0.14	ns
AIx75HR (%)	0.29	ns

GFR glomerular filtration rate, *HR* heart rate, *SBP* peripheral systolic blood pressure, *DBP* peripheral diastolic blood pressure, *AoSBP* aortic systolic blood pressure, *AoDBP* aortic diastolic blood pressure, *AoMBP* aortic mean blood pressure, *cIMT* carotid intima-media thickness, *PWV* pulse wave velocity, *AIx75HR* augmentation index corrected for heart rate 75 beats/min, *ns* non-significant

association between serum renalase and heart rate.

Results of multivariate analysis of factors affecting renalase level in children with glomerular kidney diseases are shown in Table 4. Proteinuria and serum triglycerides were the strongest predictors of serum renalase (beta = 0.45, p = 0.009 and beta = 0.45, p = 0.008), whereas age and pulse wave velocity were not statistically related to serum renalase. Table 5 presents the relationship between serum renalase and clinical and cardiovascular variables in the control group. In this group we found only an inverse association between renalase and age ($r = -0.37$, p = 0.02). There were no apparent associations between serum renalase and heart rate, blood pressure, or cardiovascular variables investigated.

4 Discussion

The major finding of the present study was that renalase was associated with proteinuria, lipids and central and peripheral blood pressure, and inversely related to kidney function in children with glomerulopathies. In healthy children, there was no relation between renalase and blood pressure or other vascular variables, and renalase was inversely related to age. Hence, despite the general lack of differences in renalase content between children with glomerular kidney diseases and healthy ones, other factors interacted in setting the serum renalase level in both populations. Interestingly, children with different disease entities, such as idiopathic nephrotic syndrome, IgA nephropathy, Henoch-Schönlein nephropathy, and others, had a comparable renalase level. In adult patients, renalase is enhanced in kidney diseases compared to healthy volunteers, but the patients examined had decreased GFR, which could have to do with the difference (Malyszko et al. 2011; Przybylowski et al. 2011).

In this study we found that children with glomerulopathies have a significantly higher peripheral and central blood pressure and a higher carotid intima media thickness and arterial stiffness compared to healthy children. The intima media thickness and pulse wave velocity belong to 'intermediate endpoints' that are directly related to 'hard endpoints' such as myocardial and brain infarcts or cardiovascular death in adults (Urbina et al. 2009). Central blood pressure is considered a better predictor of cardiovascular events compared to peripheral blood pressure, as the heart, kidneys, and major arteries supplying the brain, are exposed to aortic rather than brachial pressure (McEniery et al. 2014). The present findings indicate that children with glomerular kidney diseases, despite young age, normal kidney function, and relatively short disease duration, show already target-organ damage, which points to a substantial cardiovascular risk. As many as 38 of our patients had arterial hypertension, 32 were obese or overweight, 27 had hypercholesterolemia, and 24 had hypertriglyceridemia. These findings are in accord with sparse pediatric studies on the subject. Ksiazek et al. (2006), Alegría-Torres et al. (2015) have also found increased carotid intima media thickness in pediatric patients with idiopathic nephrotic syndrome. Likewise, Candan

et al. (2014) have shown increased pulse wave velocity in children with nephrotic syndrome. No such data have been available for children with IgA nephropathy or Henoch-Schönlein nephropathy.

In univariate analysis, we found an association between serum renalase and age, but this link disappeared in multivariate analysis by stepwise regression. In healthy children, opposite phenomenon was unraveled, i.e., younger children had a higher renalase level. In elderly patients with chronic kidney disease a similar association between renalase level and age has been found (Zbroch et al. 2016), which may be a consequence of lower GFR and higher blood pressure. In healthy children of the present study, serum renalase was much higher compared to that found in healthy adults in a study of Malyszko et al. (2011) (27.2 ± 5.2 *vs.* 3.9 ± 0.7 μg/mL, respectively). This higher renalase content could possibly be a consequence of increased sympathetic tone in children. Serum renalase in healthy children in a study of Taranta-Janusz et al. (2015), the median of 26.8 and IQR of 22.6–29.2 μg/mL, has been comparable with the present findings, but those authors fail to show a relation between age and renalase content.

In humans, renalase is mainly produced by kidneys. In adults, an inverse relation between serum renalase and glomerular filtration rate has been demonstrated (Malyszko et al. 2011; Przybylowski et al. 2011); the finding was confirmed in the present pediatric cohort. On the other hand, using a Western blot technique with polyclonal antibodies, Xu et al. (2005) have reported eight hemodialyzed patients who show diminished renalase expression compared to four healthy control subjects. In the present study we used an ELISA assay with a monoclonal antibody specific for renalase (Cloud-Clone Corp., Houston, TX, USA). The same kit was used in other studies (Zbroch et al. 2012, 2016; Malyszko et al. 2011; Przybylowski et al. 2011) The method used evaluates only the concentration and not activity of renalase. Xu et al. (2005) have suggested that plasma may contain a renalase inhibitor that may cause the activity to be low or negligible despite a high level of the protein, e.g., in patients with chronic kidney diseases. In contrast, Serwin et al. (2016), using a different kit to assess serum renalase (WuHan EIAab; Wuhan, China) have found no relation between GFR and renalase level in 64 adult patients of the mean age 46.3 ± 16.2 years with glomerulonephritis. Also, Taranta-Janusz et al. (2015), who used the same commercial kit to assess serum renalase as the one used in the present study, have found a significantly lower level of serum and urine renalase in 36 children with a solitary kidney compared to the control group. Those authors however do not demonstrate a relation between renalase and GFR. The lack of a relation between GFR and renalase could stem from a well-preserved kidney function in children with a solitary kidney (median GFR of 107.4 with IQR of 94.9–124.8).

The present study revealed a relation between renalase content and proteinuria in children with glomerulopathies. Not only had proteinuric patients a higher renalase content but there also was a linear correlation between daily urinary protein loss and renalase content. The relationship between renalase and cholesterol or triglyceride may also be a consequence of lipid abnormalities in proteinuric patients. Experimental mice studies have shown increasing sympathetic tone parallel with exacerbating protein loss (Camici 2007; DiBona et al. 1996; Herman et al. 1989).

Systemic lupus erythematosus (SLE) is an autoimmune disease of unclear pathogenesis (Guo et al. 2014). Lupus nephritis (LN) develops in 50–70% of adults and 37–82% of children with SLE (Szymanik-Grzelak et al. 2016). Qi et al. (2015) have found a higher renalase content in adult patients with lupus nephritis compared to the control group. In LN patients, renalase is associated with the score of SLE activity index, dsDNA titer, and erythrocyte sedimentation rate, and, akin to our observations, with daily urinary protein loss. Those authors however did not assess the sympathetic tone in patients and concluded that increased renalase is directly related to lupus activity.

The role of renalase in the pathogenesis of cardiovascular diseases remains unclear. In the present study, we found significant associations between renalase level and central and peripheral blood pressure indices. Interestingly, such relations were absent in healthy children. Similar relations have been found in adult kidney transplant recipients (Malyszko et al. 2011), but not in patients after heart transplantation (Przybylowski et al. 2011). In line with the latter, no relation between renalase and blood pressure has been found in Chinese adults (Wang et al. 2016) and in children with solitary kidney (Taranta-Janusz et al. 2015). The meaning of these inconclusive findings is unknown. As renalase is an enzyme inactivating catecholamines, rise in its content is suggestive of compensatory activity aimed at lowering elevated blood pressure. This phenomenon could be compared to elevated parathyroid hormone and fibroblast growth factor in chronic kidney disease in patients with hyperphosphatemia (Okarska-Napierała et al. 2016). Patients with glomerular kidney diseases have increased sympathetic system activity and elevated concentration of circulating catecholamines (Camici 2007; DiBona et al. 1996; Rahman et al. 1993; Herman et al. 1989; Ishii et al. 1983). The pediatric patients of this study had a higher heart rate compared to healthy peers, which could be considered as a rough indicator of increased sympathetic tone in children with glomerular diseases (Palatini 2013). Thus, one may speculate that renalase rise could be a protective response against 'catecholamine storm'. Experimental studies seem to confirm this notion. Supplementation of renalase-knock-out mice with exogenous renalase normalizes blood pressure and causes regression of myocardium fibrosis and left ventricular hypertrophy (Gu et al. 2011). Moreover, Maciorkowska et al. (2015) have shown that adult hypertensive patients have a higher renalase content compared to normotensives. These authors have also demonstrated an association between renalase level and noradrenaline and dopamine blood content in hypertensive individuals.

In the present study we found no appreciable relations between serum renalase level and cIMT or AIx75HR. We revealed just a trend toward an association between renalase and pulse wave velocity. It is unknown whether renalase plays a protective, harmful, or neutral role in the pathogenesis of vascular damage. This cross-sectional study does not lead to a final conclusion on the matter and the absence of a relation may also be due to a small patient sample or young age of patients. Likewise, Wang et al. (2016) have failed to demonstrate any relation between brachial-ankle pulse wave velocity and serum renalase in adult normotensive and hypertensive subjects. On the other hand, other studies suggest a protective role of renalase on heart. Wu et al. (2011) have found that renalase-knock-out mice are more susceptible to myocardial ischemia, and that treatment with recombinant renalase ameliorates cardiac injury. Farzaneh-Far et al. (2010) have reported that a functional missence polymorphism (C allele) in renalase (Glu37Asp) is associated with left ventricular hypertrophy, both systolic and diastolic dysfunction, poor exercise capacity, and inducible ischemia. Moreover, Przybylowski et al. (2011) have found in heart transplant recipients an inverse relation between renalase content and ejection fraction and a positive one between renalase and NYHA class.

Limitations of the present study include the heterogeneity of patients (children with different entities, different duration of disease, and diversified treatment protocols) and the absence of assessments of renalase urine content and of true sympathetic system activation. Especially, this last limitation precludes drawing final conclusions on the relation between blood pressure, sympathetic overdrive, and renalase content in children with glomerulopathies.

In conclusion, renalase content in children with glomerulopathies related to glomerular filtration rate, lipids, and proteinuria. Renalase may play a role in blood pressure elevation and arterial damage in glomerulopathies but this relation requires further exploration with alternative study designs.

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

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 33: 93–98
DOI 10.1007/5584_2017_86
© Springer International Publishing AG 2017
Published online: 8 August 2017

A Case of Acute Myeloid Leukemia with Novel Translocation t(6;11)(p22.2; q23) and Concurrent Insertion ins(11;9)(q23;p21.3p21.3)

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Abstract

We describe the case of a boy with acute myeloid leukemia with translocation t(6;11)(p22.2;q23) and insertion ins(11;9)(q23;p21.3p21.3). Translocation t(6;11)(p22.2;q23) involving the short arm of chromosome 6 has not been previously described. The LDI-PCR showed the presence of *KMT2A-MLLT3* fusion and identified the *BTN3A1* (butyrophilin subfamily 3 member A1) gene on 6p22.2 as the other *KMT2A* translocation partner. The *BTN3A1* gene has never been described in the context of acute leukemia. Although this fusion is out of frame, as the antisense strand of BTN3A1 is fused to the sense strand of *KMT2A*, the loss of heterozygosity of the *BTN3A1* gene might contribute to the malignancy of leukemic cells.

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Keywords
Acute myeloid leukemia • Child • Chromosomal translocation • FISH • *KMT2A* gene

1 Introduction

Chromosomal translocations involving the *KMT2A* (earlier *MLL*) gene located on chromosome 11 band q23 occur in approximately 15–20% of childhood acute myeloid leukemias (AML), mostly acute myelomonocytic and acute monocytic leukemias (Balgobind et al. 2011; Schoch et al. 2003). Recent studies have shown that the AML with *KMT2A* rearrangements is genetically heterogeneous. Over 120 different recurring translocations with almost 80 partner genes have been reported (Meyer et al. 2013). Although reciprocal balanced translocations are common in most hematopoietic malignancies (Balgobind et al. 2011), they rarely involve rearrangement of both parts of the *KMT2A* gene.

The *KMT2A* gene normally functions as a transcription regulator of the *HOX* genes and is essential for the normal mammalian development and hematopoiesis. The function of the *KMT2A* fusion genes and proteins is poorly understood, but it appears that the fusion proteins disrupt the ability of wild-type *KMT2A* to regulate *HOX* gene expression, leading to leukemogenesis.

Herein we describe the case of a boy with AML with novel translocation t(6;11)(p22.2;q23) and insertion ins(11;9)(q23;p21.3p21.3).

2 Case Report

The study was approved by a local Ethics Committee. All data were collected following the ethical principles for medical research involving human subjects as stated in the Declaration of Helsinki.

A 6-year-old boy with a 2 week history of joint pain, loss of appetite, and weight loss was admitted to the Department of Pediatrics, Hematology and Oncology, Public Pediatric Teaching Hospital, in Warsaw, Poland, with anemia and hyperleukocytosis. Upon admission, the patient demonstrated splenomegaly (3 cm below costal margin), but no signs of lymphadenopathy or hepatomegaly were found. Blood tests revealed a white blood cell (WBC) count of 20×10^9/L containing 66% blasts, hemoglobin concentration of 7.9 g/dL, red blood cells (RBC) count of 2.64×10^{12}/L, and a platelet count of 151×10^9/L. Bone marrow examination revealed 88% blasts that were positive for peroxidase staining (100%), as well as sudan black B (100%), periodic acid shiff (PAS) (85%), and esterase staining (100%). Those cells presented a monocytoidal nucleus, some of them were double-nuclear with a fine pink granularity of the cytoplasm. The immunophenotypic examination of bone marrow cells yielded the results consistent with the diagnosis of acute myeloid leukemia (M5 according to the French-American-British (FAB) classification system).

The patient was treated with a high-risk treatment protocol of the AML-BFM-1998 and AML-BFM-2004 interim phase studies (Creutzig et al. 2011). The induction treatment consisted of Ara-C (cytosine arabinoside), idarubicin, and etoposide (AIE block) and was complicated by bone marrow aplasia, persistent fever, stomatitis, and diarrhea; the diarrhea was of *Clostridium difficile* and *Candida albicans* etiology. A control biopsy on the 15th day of treatment revealed aplastic bone marrow. The patient achieved a complete remission on the 28th day of treatment.

During bone marrow aplasia after subsequent high-dose cytarabine and mitoxantrone (HAM) chemotherapy, one episode of fever and herpes simplex of lips was observed. Additionally, edema of the mandible was caused by teeth decay and the gangrenous disintegration of the teeth pulp.

Further treatment proceeded without major complications. Patient completed five courses of treatment. Twelve months after diagnosis, peripheral blood smear revealed 30% of blasts. The examination of bone marrow showed a relapse with the presence of 97% monocytoid blasts. According to the blood and bone marrow examinations, the diagnosis of AML-M5 relapse with laboratory features of disseminated intravascular coagulation was made. The patient was treated with the FLAG-IDA regimen (fludarabine, cytarabine, idarubicin, and filgrastim) and was eligible for bone marrow transplantation. Chemotherapy was complicated by bone marrow aplasia, stomatitis, diarrhea of *Clostridium difficile* and *Candida albicans* etiology, and an episode of one-day fever with colonization of the gastrointestinal tract by *Klebsiella pneumoniae* ESBL (−). On the 15th day of treatment, bone marrow remained aplastic without blasts. On the 34th day, the patient achieved the second morphologic remission, with the presence of single *KMT2A* (+) cells unraveled in the fluorescence in situ hybridization (FISH) examination. Further treatment with the FLAG-IDA regimen was complicated by fever with bacteriemia caused by *Escherichia coli* and a mild increase in liver transaminases.

Fifteen months after diagnosis, the patient underwent an unrelated peripheral stem cells transplant with conditioning consisting of busulfan with cyclophosphamide and thymoglobulin followed by acute graft-versus-host disease prophylaxis with cyclosporine and methotrexate. The engraftment was observed on the 12th day. No signs of the graft-versus-host disease were observed. More than 6 years after the transplant, the patient remains in complete remission.

3 Methods

3.1 In Vitro Culture and Banding Techniques

Bone marrow cells were cultured in RPMI 1640 supplemented with fetal bovine serum (Gibco-Thermo Fisher Scientific; Waltham, MA), antibiotics and heparin (Polfa Tarchomin S.A., Warsaw, Poland). After 24 h' culture with 5% CO_2 at 37 °C, cells were exposed to colcemide and harvested according to the standard protocols. Metaphases were analyzed by using G-banding and C-banding techniques. The karyotype was defined according to the International System for Human Cytogenetic Nomenclature (ISCN) (Shaffer and Tommerup 2005).

3.2 Fluorescence Hybridization In Situ (FISH)

The FISH was performed on interphase nuclei and metaphases using the LSI MLL Dual Color, Break Apart Rearrangement Probe (Vysis; Abbott Laboratories. Abbott Park, Ill). This probe consists of a 350 kb 5′ region, centromeric (between exon 6 and 8) to the *KMT2A* gene bcr (breakpoint cluster region), labeled in spectrum green, and 190 kb 3'region telomeric (between exon 4 and 6) to the bcr, labeled in spectrum red. Separate red and green signals indicate the *KMT2A* gene rearrangement; a co-localization of green-red signals indicates the germline *KMT2A* configuration. For the detection of translocation partner chromosomes, the human whole chromosome painting probes for chromosomes 9 and 11 (XCP; MetaSystems GmbH, Altlussheim, Germany) were used. The image acquisition was performed with a fluorescence Zeiss Axio Imager A1 microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Images were analyzed using the MetaSystems Ikaros and Isis image analysis systems (MetaSystems Hard & Software GmbH, Altlussheim, Germany).

3.3 PCR and Gene Sequencing

The method for long-distance inverse PCR (LDI-PCR) has been previously described in detail (Meyer et al. 2005). Briefly, genomic DNA was extracted and digested with the restriction enzyme BamHI. The sample was self-ligated

in the presence of ligase T4 to form the DNA circles, which were subsequently amplified with different combinations of primers annealing to the *KMT2A* sequences. The PCR amplimers were separated on agarose gel and subjected to DNA sequence analysis.

4 Results and Discussion

Cytogenetic examination of bone marrow cells at diagnosis demonstrated a normal male karyotype (46,XY) in 14 bone marrow cells, while FISH examination showed a rearrangement of the *KMT2A* gene in 85% of the interphase cells. Bone marrow reexamination at relapse (Fig. 1A) revealed 45,XY,t(6;11)(p21.3;q23),der(8)t(8;17)(p2?1;q11.2),-12,der(17)t(12;17)(q1?3;q11.2). FISH examination at relapse with MLL probe (Fig. 1B and C) revealed a split signal in both interphase and metaphase cells, indicating a rearrangement of the *KMT2A* gene (11q23). FISH examination using whole chromosome painting probes (Fig. 1D), revealed translocation t(6;11) and insertion of a part of the short arm of chromosome 9 into the long arm of chromosome 11 – ins (11;9). The insertion was not seen on metaphases (cryptic aberration). The LDI-PCR showed the presence of *KMT2A-MLLT3* and *BTN3A1-KMT2A* fusions (Fig. 2), define the breakpoint on chromosome 9: 9p21.3 (*MLLT3* gene) and verified the breakpoint on chromosome 6: 6p22.2 (*BTN3A1* gene) initially described in karyotype as a 6p21.3.

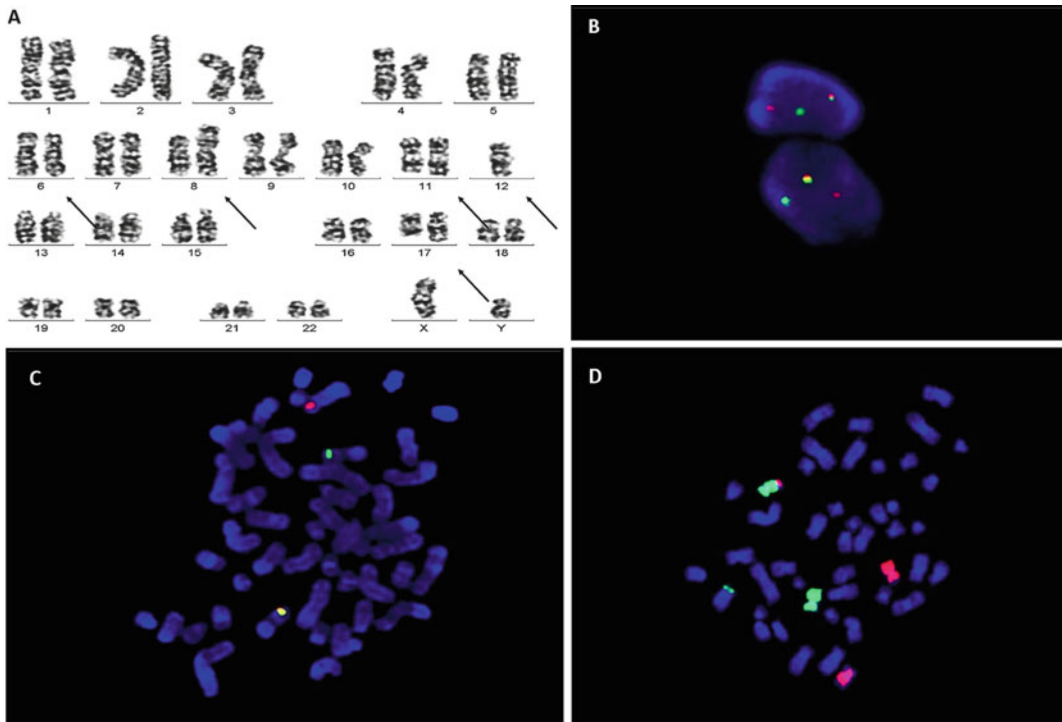

Fig. 1 (**A**) G-banded karyotype showing: 45,XY,t(6;11)(p21.3;q23),der(8)t(8;17)(p2?1;q11.2),-12,der(17)t(12;17)(q1?3;q11.2). *KMT2A* split-signal FISH on interphase cells (**B**) and metaphase cells (**C**) confirming the *KMT2A* gene rearrangement and showing 5′ proximal (*green*) signal on derivative chromosome 11 and 3′ distal (*red*) signal on derivative chromosome 6. (**D**) Examination with human whole chromosome painting probes (XCP, MetaSystems) for chromosome 9 (*red*) and chromosome 11 (*green*) showing normal single copies of chromosome 9 (*red*) and chromosome 11 (*green*), as well as the transfer of a proximal fragment of the long arm of chromosome 11 (*green*) into the short arm of chromosome 6 – translocation t(6;11) and insertion ins (11;9)

```
A
MLL wt  (+)    GATAAAGAGATAGTGTGGTGGCTCACATCTATAATCCCAGCACTTTGGGGGGCCAAGGCG
               IIIIIIIIIIIIIIIIIIIIIIIIIIIIIII
MLL-MLLT3      GATAAAGAGATAGTGTGGTGGCTCACATCTAAAAGACAAATTACTTTGCTTTTTGTGTTT
                                             IIIIIIIIIIIIIIIIIIIIIIIIIIIIII
MLLT3 wt(+)    AACAACTCACTTCATAATGAGTTATAATGAAAAAGACAAATTACTTTGCTTTTTGTGTTT


B
BTN3A1 wt(-)   ATGAAAAAAAGCATAAAGGAATACAAGTTTAAGTAGTGGTTCATGCCTGTAATCCTTGCA
               IIIIIIIIIIIIIIIIIIIIIIIIIIIIII
BTN3A1-MLL     ATGAAAAAAAGCATAAAGGAATACAAGTTTCTGGAGACCAGCCTGGGCAGCACAGTGACA
                                             IIIIIIIIIIIIIIIIIIIIIIIIIIIIII
MLL wt  (+)    CAAGGCGGGAGGATCACTTGAGGGCAGGAGCTGGAGACCAGCCTGGGCAGCACAGTGACA
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Fig. 2 Genomic breakpoint analysis of the complex *KMT2A (MLL)* rearrangement. (**A**) *KMT2A-MLLT3* genomic breakpoint sequence alignment with respective wild type sequences; this is in-frame fusion. (**B**) *BTN3A1-KMT2A* genomic breakpoint sequence alignment with respective wild type sequences; this is out-of-frame fusion as the *BTN3A1* antisense strand (−) is fused to the *KMT2A* sense strand (+)

The t(9;11)(p21;q23) resulting from *KMT2A-MLLT3* fusion is the most frequent aberration in *KMT2A*-rearranged AML and fourth most common in AML after t(8;21)(q22;q22), t(15;17)(q22;q21) and inv(16)/t(16;16)(p13;q21). It was the first reported translocation typical for AML by Hagemeijer et al. (1982). The t(9;11)(p21;q23) is very subtle and it may be overlooked in case of unsatisfactory or fair chromosome morphology. In such a case, the use of FISH technique is essential. A more useful technique is the use of FISH on previously G-banded slides containing metaphases. The t(9;11) may arise through a two-way translocation, but it also could take place through the more complex mechanisms, such as three-way translocation t(9;11;Var) involving a (variable) third chromosome. In these complex translocations, *KMT2A-MLLT3* fusion is crucial for leukemogenesis. Translocation t(6;11)(p22.2;q23) involving the short arm of chromosome 6 has not been previously described. This translocation was confirmed by the LDI-PCR examination that identified the *BTN3A1* (butyrophilin subfamily 3 member A1) gene on 6p22.2 as *KMT2A* translocation partner. Although this fusion is out of frame, as the antisense strand of *BTN3A1* is fused to the sense strand of *KMT2A,* the loss of heterozygosity of the *BTN3A1* gene may also contribute to the malignancy of leukemic cells. The *BTN3A1* gene has never been described in the context of acute leukemia.

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

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 33: 99–100
DOI 10.1007/978-3-319-65469-0
© Springer International Publishing AG 2017
Published online: 13 April 2017

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