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Neuroscience and Respiration

Mieczyslaw Pokorski *Editor*

Environmental Biomedicine

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Preface

The book series *Neuroscience and Respiration* presents contributions by expert researchers and clinicians in the field of pulmonary disorders. The chapters provide timely overviews of contentious issues or recent advances in the diagnosis, classification, and treatment of the entire range of pulmonary disorders, both acute and chronic. The texts are thought as a merger of basic and clinical research dealing with respiratory medicine, neural and chemical regulation of respiration, and the interactive relationship between respiration and other neurobiological systems such as cardiovascular function or the mind-to-body connection. The authors focus on the leading-edge therapeutic concepts, methodologies, and innovative treatments. Pharmacotherapy is always in the focus of respiratory research. The action and pharmacology of existing drugs and the development and evaluation of new agents are the heady area of research. Practical, data-driven options to manage patients will be considered. New research is presented regarding older drugs, performed from a modern perspective or from a different pharmacotherapeutic angle. The introduction of new drugs and treatment approaches in both adults and children also is discussed.

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

Neuromolecular aspects relating to gene polymorphism and epigenesis, involving both heritable changes in the nucleotide sequence and functionally relevant changes to the genome that do not involve a change in the nucleotide sequence leading to respiratory disorders, will also be tackled. Clinical advances stemming from molecular and biochemical research are but possible if the research findings are translated into diagnostic tools, therapeutic procedures, and education, effectively reaching physicians and patients. All that cannot be achieved without a multidisciplinary, collaborative, bench-to-bedside approach involving both researchers and clinicians.

The societal and economic burden of respiratory ailments has been on the rise worldwide leading to disabilities and shortening of life span. COPD alone causes more than three million deaths globally each year. Concerted efforts are required to improve this situation, and part of those efforts are gaining insights into the underlying mechanisms of disease and staying abreast with the latest developments in diagnosis and treatment regimens. It is hoped that the books published in this series will assume a leading role in the field of respiratory medicine and research and will become a source of reference and inspiration for future research ideas.

I would like to express my deep gratitude to Martijn Roelandse and Tanja Koppejan from Springer's Life Sciences Department for their genuine interest in making this scientific endeavor come through and in the expert management of the production of this novel book series.

Opole, Poland Mieczyslaw Pokorski

Volume 10: Environmental Biomedicine

The book *Environmental Biomedicine* presents novel experimental achievements in the many facets of environment safety, air pollution, and health preventive medicine. The chapters tackle the detrimental health effects and prevention of environmental air pollution in car traffic congested areas of cities, but also focus on the spread of air-borne infectious agents, and on the effects of cigarette smoke, an inescapable theme when dealing with air pollution. The oral orifice is the entry way for ambient air, and thus for its biological and chemical content during breathing. Understandably, pollution is a major detriment to the respiratory health. The book highlights other, less recognized, but as important areas of current research as metabolic and proinflammatory effects of air pollution, or oxidation-related effects on skin cells, another tissue directly exposed to air content. Increased knowledge of air-borne health hazards facilitates the development of preventive measures and shifts the emphasis to avoiding problems before they occur.

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The Role of Oxidation in FSL-1 Induced Signaling Pathways of an Atopic Dermatitis Model in HaCaT Keratinocytes

I. Koren Carmi, R. Haj, H. Yehuda, S. Tamir, and A.Z. Reznick

Abstract

Oxidative stress (OS) is common in inflammatory conditions and may be important in atopic dermatitis (AD) etiology. The aim of this project was to study the involvement of oxidation in FSL-1 (deacylated lipoprotein)-triggered signaling pathways leading to AD-typical cytokine expression in HaCaT keratinocytes. HaCaT keratinocytes, pretreated with the inhibitor to OS N-acetylcysteine (NAC), were exposed to FSL-1, a stimulator of AD-related cytokines. Cytokines expression was studied by real time polymerase chain reaction (PCR); nuclear factor-kappa B (NF-κB) and p38 mitogen activated protein kinase (MAPK) activities were studied by western blotting; and the oxidative state of cells was determined by the dichlorofluorescein (DCF) assay. We found that endogenous OS in keratinocytes appeared 4 h after FSL-1 administration. OS activated NF-κB, but not p38 MAPK, and the inhibition of OS reduced FSL-1 induced interleukin (IL) 33, thymic stromal lymphopoietin (TSLP) and TNFα mRNA expression. We conclude that FSL-1 triggers an OS reaction in HaCaT keratinocytes, which is probably a secondary event affecting the expression of specific AD typical cytokines, possibly through the NF-κB pathways. This role of OS in the inflammatory response in AD is worth further investigating.

Keywords

Inflammation • N-acetylcysteine • Nuclear factor-kappa B • p38 mitogen activated protein kinase • Thymic stromal lymphopoietin

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1 Introduction

1.1 Atopic Dermatitis (AD)

AD is a chronic and relapsing inflammatory skin disease characterized by dry, itchy, eczematous skin lesions which are susceptible to cutaneous bacterial, viral, and fungal infections. In the past decades, its prevalence has increased in the developed countries affecting up to 25 % of all children and 1–10 % of adults worldwide (Novak and Simon 2011). AD causes a significant impairment in the patient's quality of life and treatments approaches are limited to symptomatic, unspecific anti-inflammatory or immunosuppressive agents (Boguniewicz and Leung 2010).

The homogenous symptoms of AD imply a shared common disease, but recent research efforts have revealed the complex nature of this fascinating disease, which nonetheless cannot be considered to be a uniform entity. AD frequently starts in early infancy, termed early-onset AD, as opposed to late-onset AD, which develops in adulthood (Terui 2009). In about 70–80 % of patients, AD takes on the 'extrinsic' form, which is associated with increased IgE and eosinophil levels in the serum, while in the remaining patients AD has the 'intrinsic' form, characterized by normal IgE levels. Several studies have shown that, at the age of 5 years, 50 % of the children with AD have developed other atopic and allergic respiratory diseases – asthma, allergic rhinitis, and sensitization to inhalant allergens (Patrizi et al. 2011). Thus, AD is often the initial step in the so-called "atopic march" (Lenung et al. 2004).

Two hypotheses propose the cause underlying the disease. One suggests that the primary defect is genetic immunologic disturbance, with an epidermal dysfunction which is induced by local inflammation. This hypothesis is supported by the development of AD in a bone marrow recipient after the engraftment of hematopoietic stem cells from an atopic donor (as he receives the 'atopic' immune cells of the atopic donor). The second hypothesis, which is supported by strong evidence in recent studies, claims that a primary structural abnormality of the stratum corneum (SC) underlies the pathogenesis of AD (Terui 2009).

Data that has accumulated over the past years has indicated the ability of epithelial cells to initiate and direct the immune response in allergic skin diseases. Upon activation, keratinocytes will initiate a process of alerting and activating the immune system in a way that will predominantly drive a T_H2 response. Thymic stromal lymphopoietin (TSLP), IL25 and IL33 constitute a keratinocyte-derived triad of cytokines, which collectively drives T_H2 polarization through complimentary and sometimes synergistic mechanisms.

TSLP was found to be highly expressed in epithelial cells, especially keratinocytes from patients with AD (Carmi-Levy et al. 2011) as well as in AD lesions and bronchial epithelial cell of asthmatic patients (Tuan Vu et al. 2010). Tissue-specific overexpression of TSLP in mouse skin induced an AD-like phenotype and variants of TSLP and its receptor have been found to be associated with the risk of AD (Carmi-Levy et al. 2010). TSLP was recently found to be produced following skin tape stripping, which induces one of the first features of AD, namely the disruption of the skin epithelial barrier (Carmi-Levy et al. 2011) and may be the link between epithelial barrier dysfunction and T_H2 immune polarization in AD.

1.2 Oxidative State

Oxidative stress has a major role in AD. Due to the skin's function as an interface between the body and the environment, it is chronically exposed to both endogenous and environmental pro-oxidant agents, leading to the harmful generation of reactive oxygen species (ROS). ROS are involved in the damage of cellular constituents, such as DNA, cell membrane lipids, or proteins and can act as secondary messengers in the induction of several biological responses, such

as the activation of the NF-κB signal transduction pathway, the generation of cytokines, and the modulation of other signaling pathways (Tsukahara et al. 2003). Peroxides have been shown to be produced in keratinocytes following UVB activated MAPK signal transduction pathways (Peus et al. 1999). In another study, UVA stimulation was found to induce ROS production by activation of NADPH oxidase probably in the mitochondria (Valencia and Kochevar 2008).

1.3 P38 MAPK

The MAPKs, which integrate and process various extracellular signals, are primary components of the intracellular signaling circuitry. Four variants of p38 MAPK have been identified (α, β, γ, and δ). p38α is perhaps the most physiologically relevant kinase involved in inflammatory responses, with a central role in the expression of pro-inflammatory cytokines and in immune cell proliferation and differentiation.

1.4 NF-κB

The master regulator of inflammation is the transcription factor NF-κB. It is also considered to be a prototypical example of sensitivity to oxidative stress. Non-activated NF-κB is composed of p50 or p52, p65 (REL-A, REL-B, c-REL) and the inhibitor IκB (IκBα, IκBβ, IκBε, bcl-3), while the complex p50-REL-A and the inhibitor IκBα are most common. In the classical pathway, NF-κB is activated *via* phosphorylation of IκB at two conserved serine residues in the N-terminus (Ser32, Ser36) by IKK (IκB kinases – composed of IKK1, IKK2 and NEMO subunits), followed by ubiquitination and proteolysis of its bound inhibitor – IκB. This allows for the migration of the liberated NF-κB into the nucleus and its activation as a transcription factor. In the canonic activation mechanism, phosphorylation of IκB, its degradation and the activation of NF-κB results in the transcription of the inhibitor IκB, thus IκB mRNA expression indicates NF-κB activation (Perkins 2006).

In the present research, HaCaT human keratinocytes were treated with FSL-1, a diacylated lipoprotein that has been found to upregulate the expression and secretion of TSLP in human keratinocytes (Tuan Vu et al. 2010). TSLP expression in keratinocytes is a phenomenon that is specific to atopic dermatitis (Carmi-Levy et al. 2010; Tuan Vu et al. 2010). Therefore, this system was chosen to study the involvement of ROS in AD-related cytokine expression in keratinocytes.

2 Methods

2.1 HaCaT Keratinocytes

The human immortal keratinocyte cell line, HaCaT, was grown in Dulbecco's Modified Eagle's Medium (DMEM, Biological Industries, Beit Haemek, Israel) supplemented with 2 mM L-glutamine (Biological Industries, Beit Haemek, Israel), 10 % fetal calf serum (Sigma-Aldrich, Rehovot, Israel), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Biological Industries, Beit Haemek, Israel) at 37 °C, with 5 % CO_2. Viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich, Rehovot, Israel). In experiments, cells were seeded in DMEM in six wells plates and were pretreated with/without 2 mM NAC (Sigma-Aldrich, Rehovot, Israel) for 60 min followed by the administration of 1 μg/ml FSL-1 for indicated periods of time.

2.2 Induction of AD-Related Cytokine Reaction

Diacylated lipoprotein, FSL-1 (Fibroblast-Stimulating Lipopeptide-1, InvivoGen, Toulouse, France) is a synthetic lipoprotein that represents the N-terminal part of the 44-kDa lipoprotein LP44 of *Mycoplasma salivarium*.

Mycoplasmal lipoproteins, such as FSL-1 and MALP-2, contain a lipolyated N-terminal diacylated cysteine residue, whereas bacterial lipoproteins contain a triacylated one. This structural difference plays a crucial role in the initial recognition of microbial lipoprotein by the host innate immune system.

2.3 Real Time PCR

mRNA was isolated using TRI Reagent (Sigma, Rehovot, Israel) and cDNA was prepared using the Verso cDNA kit (Thermo Scientific, US) and programmed 42 °C for 60 min, 50 °C for 30 min, and 95 °C for 2 min. The cDNA of the mRNA transcript was amplified by spectrofluorometric thermal cycler (Rotor-Gene^{TM} 6000, Corbett research, Mortlake, Australia) using KAPA Sybr fast (KAPA Biosystems, Cape Town, South Africa) with specific primers for: GAPDH (housekeeping – to which every gene of interest was normalized -5′ → 3′ CGACCACTTTGTCAAGCTCA, TGTGAGGAGGGGAGATTCAG), IκBα (5′ → 3′ CTGTGATCACCAACCAGCCAGA, GTAGCCATGGATAGAGGCTAAG), TSLP (5′ → 3′ TGCCTGCGGCTCTAGCTTGC, AGCCCACTGACTGCTCCCCT), IL33 (5′ → 3′ CCTCAAATGAATCAGGTGACGGTGT, ACAAAGAAGGCCTGGTCTGGCA), IL25 (5′ → 3′ CCTGCTAGGCCCAACCGCCA, GGGGTCCATGTGGGAGCCTGT) and TNFα (5′ → 3′ GTGATCGGCCCCCAGAGGGA, CACGCCATTGGCCAGGAGGG). Results were represented as fold change of the control (without treatment at each time point).

2.4 Protein Analysis

Proteins from cells lysed in a solution containing CelLytic Lysis Buffer (Sigma, Rehovot, Israel) and Protease Inhibitor Cocktail (Sigma, Rehovot, Israel), were separated on a standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotted on nitrocellulose membranes. Blots were exposed overnight to primary monoclonal antibodies (separately) for: α-tubulin (Santa Cruz Biotechnology, Dallas, Texas, US), p38 MAPK (R&D systems, Minneapolis, US), phospho-p38 MAPK (T180/Y182 R&D systems, Minneapolis, US), IκBα (Santa Cruz Biotechnology, Dallas, Texas, US) and phospho-IκBα (S32/S36 Cell Signaling, Danvers, Massachusetts, US) at room temperature. The secondary antibodies – anti goat or anti rabbit (according to the primary antibody used) conjugated to horse-radish peroxidase (Jackson Immuno-Research, West Grove, Pennsylvania, US) were used. Detection was performed by enzyme-linked chemiluminescence (ECL) (Biological Industries, Beit Haemek, Israel), using ImageQuant LAS 4000 Digital Imager System (GE Healthcare, Buckinghamshire, UK). Protein quantities were determined by densitometry and analyzed using Total Lab Software (version V2006C, Nonlinear Dynamics, University of Leicester, UK). Results are represented as fold change relative to the control (without treatment at time zero).

2.5 Dichlorofluorescein (DCF) Assay

The DCF assay measures the level of oxidative stress. Cells grown to 50 % confluence on cover glass in 24-well plates were detected with the nuclear DNA-staining fluorescent dye Hoechst (1.5 μg/ml, incubation for 30 min). These cells were incubated with 10 μM dichlorofluorescein diacetate (CM-H_2DCFDA, Invitrogen, Toulouse, France) for 60 min at 37 °C in the dark for the internalization of this chemical, which fluoresces upon oxidation. Subsequently, cells were pre-exposed to the OS inhibitor, NAC, for 60 min before the stimulation with 1 μg/ml FSL-1. The difference in the fluorescence was quantitated by fluorescence microscopy and the average spectrometric optical density (OD) values were calculated. Results are represented as fold-change from the control (without treatment at each time point).

3 Results

3.1 FSL-1 Stimulates ROS Production Which Is Reduced by OS Inhibition

The induction of ROS by FSL-1 treatment of keratinocytes was verified by a DCF assay. After HaCaT cells were stimulated with 1 μg/ml FSL-1, ROS levels increased (Fig. 1a) by 13 %, but only at 4 h (tested in 2 h and 6 h as well) following FSL-1 treatment, in comparison to the negative control (without treatment at each time point). At 4 h, when the keratinocytes were pretreated with 2 mM NAC, the FSL-1 induced ROS (at 4 h) decreased by 27 %, as compared to the negative control (Fig. 1a, b).

3.2 FSL-1 Does Not Induce Phosphorylation of p38 MAPK Through OS

In order to learn if FSL-1 affects the p38 MAPK pathway by means of OS, HaCaT cells were pretreated with the antioxidant NAC (2 mM) for 60 min before exposure to FSL-1 (1 μg/ml 10–120 min). The western blot showed that FSL-1 induced p38 MAPK phosphorylation, but this induction was not significantly inhibited by NAC (Fig. 2a, b), implying that OS is not involved in FSL-1 triggered phosphorylation of p38 MAPK.

3.3 OS Is Not Involved in FSL-1 Induced NF-κB Activity Immediately After FSL-1 Administration

The participation of OS in FSL-1 induced NF-κB activity was investigated. Western blots of NF-κB activation revealed that FSL-1 (1 μg/ml) induced NF-κB activity, which was not inhibited by NAC.

Although lower levels of phospho-IκBα at 30 min, lower production *de novo* of IκBα at 120 min and lower IκBα mRNA expression, as compared to levels obtained from FSL-1 alone, were observed (Fig. 3a–d), no inhibition of

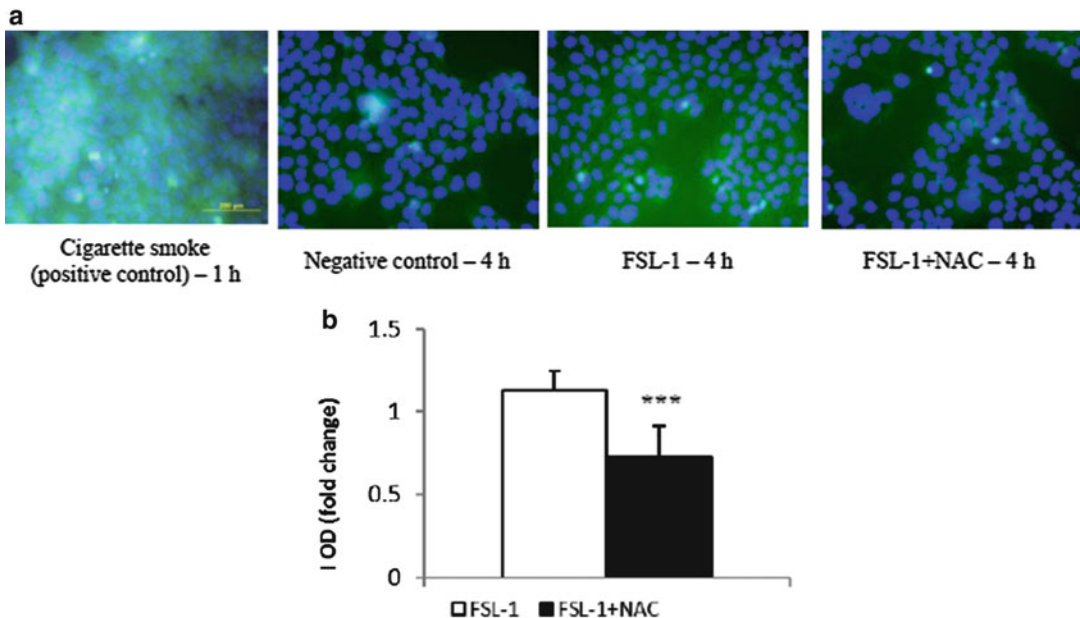

Fig. 1 **FSL-1 induces ROS which is inhibited by NAC.** HaCaT keratinocytes were pretreated with 2 mM NAC 1 h prior to stimulation with FSL-1. ROS levels were determined using DCF, microscopy imaging (**a**) and computerized quantifying, presented relatively to control without treatment (**b**). Number of cells tested: FSL-1 = 350; FSL-1 + NAC = 180. ***$p < 0.0005$; Data are means ± SE

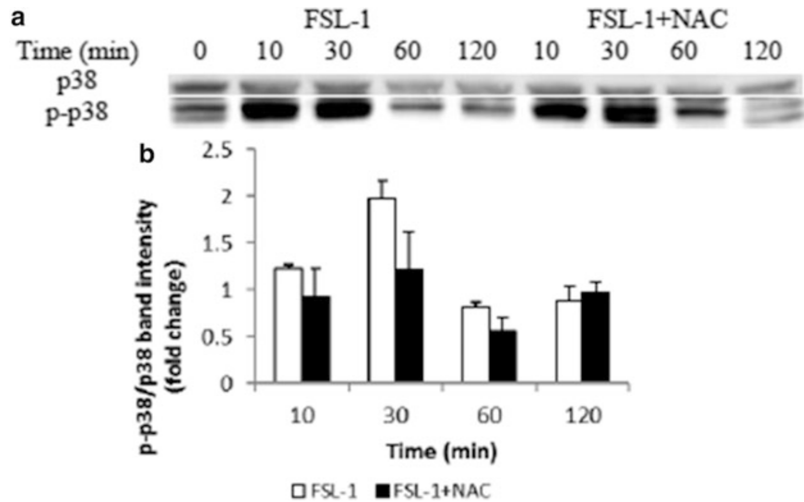

Fig. 2 The antioxidant NAC does not inhibit FSL-1-induced phosphorylation of p38 MAPK. HaCaT keratinocytes were treated with 2 mM NAC prior to stimulation with FSL-1 (1 μg/ml). Representative western blot (**a**) and calculated phospho-p38/p38 ratios (**b**) of time dependent p38 and phospho-p38. Number of wells tested: FSL-1 = 6–8; FSL-1 + NAC = 2–3. Data are means ± SE

NF-κB activation by NAC took place. This is concluded from the lower levels of IκBα 10–30 min after FSL-1 administration (Fig. 3b), which should have been higher in case of NF-κB activation inhibition. NAC is able to decrease the level of phospho-IκBα by its reduction and not only by sequestering OS. In summary, these results indicate that OS was not apparent immediately after FSL-1 administration.

3.4 Inhibition of OS Reduced FSL-1 Induced TSLP, TNFα and IL33 mRNA Expression

The effect of OS on the induction of AD-related cytokines triggered by FSL-1 was also studied. Keratinocytes were tested for mRNA expression of the AD typical cytokines by real time PCR at different time periods, following FSL-1 stimulation, with and without 2 mM NAC pretreatment (Fig. 4).

NAC had no significant effect on FSL-1 induced IL25, but it did have an inhibitory effect on FSL-1-induced TSLP, IL33 mRNA (at 4 h) and TNFα expression (at 2 and 6 h) after FSL-1 administration, indicating the participation of OS in the mRNA expression of these AD-related cytokines.

4 Discussion

The aim of this research was to study the role of oxidative stress in the signal transduction pathways of AD. The *in vitro* model chosen was HaCaT keratinocytes stimulated by FSL-1, a diacylated lipoprotein, which was found to upregulate the expression and secretion of TSLP in primary human keratinocytes through ligation to TLR2 or TLR6 (Tuan Vu et al. 2010). Furthermore, FSL-1 stimulation induces a MyD88-dependent signaling cascade leading to the activation of NF-κB and the production of proinflammatory cytokines. FSL-1 has also been found to rapidly activate IκB phosphorylation in keratinocytes and to bring about the high expression of TNFα in macrophages and NF-κB in TLR2/TLR6 transfected HEK293 cells (Zhaoa et al. 2010).

In this study, it was found that FSL-1 induced the production of endogenic oxidative species, besides activating NF-κB, p38 MAPK and brought about an increase in the expression of IL33, TSLP, and TNFα cytokines. The induction of cytokine expression was inhibited by an OS inhibitor, NAC, implying the involvement of OS in certain AD-related pathways in keratinocytes.

FSL-1 induced the production of ROS in keratinocytes. Since the time period required for a significant elevation of the oxidation level

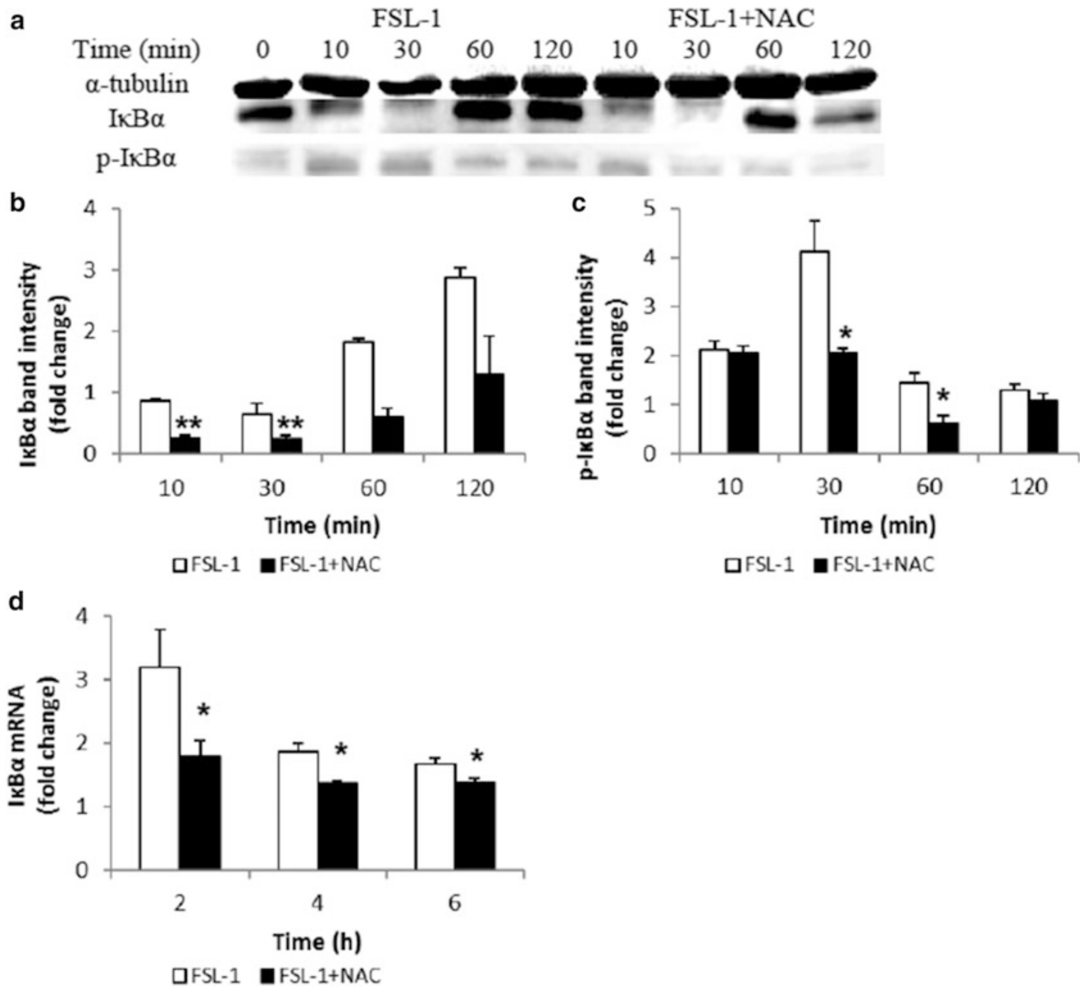

Fig. 3 NAC inhibits phosphorylation, expression and resynthesis of IκBα in FSL-1 by means of its reduction and not by OS sequestering. HaCaT keratinocytes were treated with and without 2 mM NAC prior to stimulation with FSL-1 (1 μg/ml). Time dependent IκBα and phospho-IκBα levels are presented in representative western blot (**a**); and protein levels were quantified by densitometry and presented relative to negative control (without treatment). Relative protein levels of IκBα (**b**) and phospho-IκBα (**c**). Number of wells tested: FSL-1 = 4–6; FSL-1 + NAC = 2–3. Time dependent IκBα mRNA expression was measured using real time PCR and is presented normalized to the housekeeping gene (GAPDH) and relative to negative control (without treatment) at each time point (D). Number of wells tested: FSL-1 = 9–12; FSL-1 + NAC = 3. *$p < 0.05$, **$p < 0.005$ relative to FSL-1 alone. Data are means ± SE

is longer (4 h) than that observed after stimulation with other triggers of OS, e.g. cigarette smoke, the reaction to FSL-1 is probably a secondary reaction. It is well known that OS is related to inflammation of all types, as cells of the immune system produce and secrete different kind of enzymes, ROS, and nitric oxide species (NOS) to fight intruders, but also in the case of sterile inflammation, meaning when no pathogens are involved (Kim et al. 2011). Keratinocytes, which are the body's first line of defense and barrier from the outside world, are part of the innate immune system and are also able to produce ROS. In the case of AD, oxidation may be the fuel of the vicious cycle that is the basis of the disease chronicity.

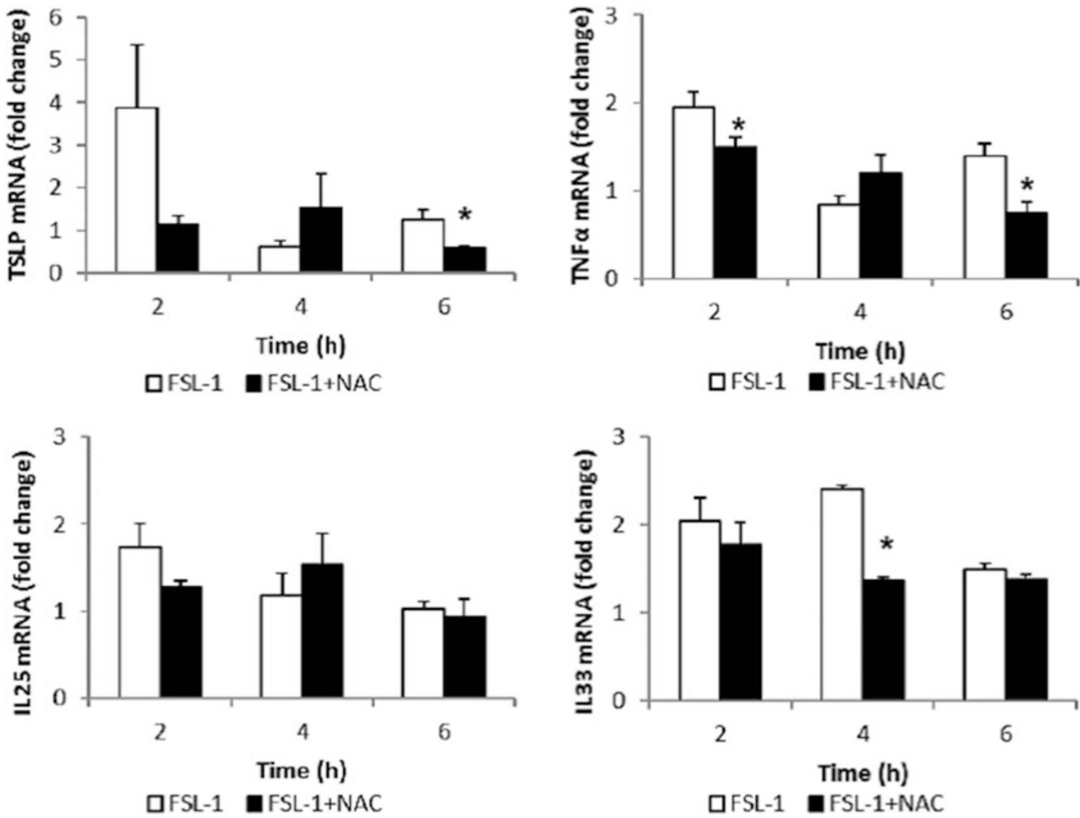

Fig. 4 **NAC decreases FSL-1-induced mRNA expression of TSLP, TNFα, and IL33**. HaCaT cells were pretreated with 2 mM NAC before stimulation with FSL-1 (1 μg/ml). Time dependent mRNA expression was measured using real time PCR and is presented as normalized to the housekeeping gene (GAPDH) and relative to the negative control (without treatment) at each time point. Number of wells tested: FSL-1 = 3–14; FSL-1 + NAC = 3. *$p < 0.05$ relative to FSL-1 alone. Data expressed as means ± SE

The observed rise in the prevalence of AD cannot be attributed to genetics and it has been hypothesized that it may be due to the increase in ROS generated from environmental pollution and solar radiation. In Nagoya City, where nitrogen oxide concentration is the highest in Japan, an increase in the incidence of AD patients was detected. It was also reported that there is a greater number of AD patients in urban areas than in rural districts, again pointing to the possible connection between environmental oxidation and AD (Niwa et al. 2003).

FSL-1 does not induce phosphorylation of p38 MAPK through OS shortly after its administration; meaning, p38 MAPK does not recruit NF-κB, which in turn leads to cytokine production in the cascade resulting from OS, as was hypothesized and indicated in literature (Kumar et al. 2003; Gochman et al. 2011; Kaisari et al. 2013).

OS does not show any apparent relation to the NF-κB pathways immediately after FSL-1 administration, probably since no rise in OS was observed shortly after FSL-1 administration. A series of investigations showed that oxidative stress activates NF-κB in different types of cells, but not in all. In addition, several recent studies have indicated that oxidants, such as hydrogen peroxide or peroxynitrite, repressed NF-κB activation in several cell systems *in vitro* (Loukili et al. 2010).

An indication for NF-κB relevance to AD is exampled in a research that administered topical NF-κB decoy oligodeoxynucleotides in NC/Nga

atopic mouse model twice a month and found a significant reduction in clinical skin condition and a marked improvement of histological findings (Morishita et al. 2004). Another indication of the relevance of NF-κB in AD is the finding that many cytokines related to inflammation in general and to AD in particular, seem to be regulated by NF-κB (it promotes the transcription of TNFα, T_H2 cytokines, and TSLP which is known to be an important factor in atopic diseases) (Moon and Kim 2011). In this study, it was found that the antioxidant NAC was apparently able to sequester the induced ROS, thus preventing the OS from causing up-regulation of TSLP, IL33, and TNFα (as mentioned previously, TSLP and IL33 are known AD-related cytokines, while TNFα is an inflammatory cytokine) mRNA expression. Possibly, NAC prevented the increase in cytokine expression by means of NF-κB inhibition.

Expression of IL25 (which is related to the T_H2 immune response and was detected in human AD lesions and in a murine model for allergic airway disease) was not found to increase as a consequence of FSL-1 employment. The explanation, as was found upon further investigations, is that IL25 is produced by dendritic cells within the dermis of patients and not by keratinocytes (Hvid et al. 2011).

These triggered pathways may lead to the additional production of AD-typical cytokines in what may be a vicious cycle, in which cell components are damaged and chronic atopic disease develops. An example of this possibility is the immunomodulatory T_H2-associated cytokine, IL33, which activates NF-κB in a murine cutaneous model (Pushparaj et al. 2009). Another possible reactivation pathway may be through TNFα and its receptor, which are known together to activate NF-κB.

The role of OS in AD is important, since oxidation may be the instigator of the vicious cycle that is responsible for the chronicity of this disease. The elucidation of the OS-related processes contributing to AD may reveal targets that may be relevant in new, more efficient treatments, with fewer side effects. Such treatments could be alternatives to the corticosteroids used today, or to the expensive, and yet to be approved biological therapy (Guttman-Yassky et al. 2013).

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

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Epidemiological and Clinical Reasons for Vaccination Against Pertussis and Influenza in Pregnant Women

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Abstract

Vaccinations in pregnancy are an important aspect of prenatal care for improving both maternal health and neonatal outcomes. Despite the fact that protection against some infectious diseases for pregnant women can be easily provided through immunizations, current coverage rates are low. Two vaccines are notably recommended during pregnancy: influenza and the combined tetanus, diphtheria and acellular pertussis (Tdap) vaccine. In this review the authors discuss current recommendations for vaccination against pertussis and influenza in pregnant women in terms of epidemiological, clinical, and immunological reasons, taking into account safety and effectiveness. Promoting patients' awareness about pertussis and influenza and encouraging general practitioners, nurses and obstetricians to recommend the pertussis booster and influenza vaccine will hopefully increase the number of pregnant women who choose to become vaccinated.

Keywords

Prophylaxis • Maternity • Whooping Cough

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Abbreviations

ACOG	American College of Obstetricians and Gynecologists
ACIP	Advisory Committee on Immunization Practices Centers for Disease Control and Prevention
CDC	Centers for Disease Control and Prevention
DTaP	tetanus diphtheria and acellular pertussis vaccine with not reduced doses of antigens, used in infants and children
ECDC	European Centre for Disease Control and Prevention
FDA	Food and Drug Administration
ICU	Intensive Care Unit
Tdap	tetanus diphtheria and acellular pertussis vaccine with reduced doses of antigens, used in adolescents and adults
VAERS	Vaccine Adverse Event Reporting System
VSD	Vaccine Safety Datalink
WHO	World Health Organization

1 Introduction

Effective protection against some infectious diseases for pregnant women and their children can be provided through immunization administered during pregnancy. This means that vaccinations during pregnancy are an important aspect for both maternal and prenatal care, improving the health safety of neonates. Two vaccines are notably recommended during pregnancy: the influenza vaccine and the combined tetanus, diphtheria and acellular pertussis (Tdap) vaccine. We present the current recommendations for immunization against pertussis and influenza in pregnant women in terms of epidemiological, clinical, and immunological reasons. The safety and effectiveness issues for both women and infants are also discussed.

2 Influenza

2.1 Epidemiology and Clinical Course of Influenza in Pregnant Women and Their Children

Influenza is one of the most prevalent viral diseases and a major cause of morbidity and mortality in many regions around the world. The World Health Organization (WHO) estimates between 330 million and 1.575 billion people may suffer from influenza each year, while between 500 thousand and one million people die annually due to influenza and its complications (WHO 2009).

Although the incidence rates of influenza are similar among pregnant and non-pregnant women, pregnant women have an increased risk of experiencing a severe and complicated course of influenza. Factors increasing the severity and risk of complications are associated with physiological changes mainly occurring during the third trimester of pregnancy, including: changes in the immune system (cellular immunodeficiency, selective suppression of Th1 cells), increased cardiac ejection fraction, increased oxygen consumption, and reduced lung volume (Creanga et al. 2010; Steinhoff et al. 2010; Puck et al. 1980).

A severe course of influenza in pregnancy was first reported during the pandemic of 1918, when 1,350 cases in pregnant women, who had an influenza-like illness, were evaluated and when pneumonia complicated 43 % of the cases. In 52 % of these patients, pregnancy was prematurely terminated. The mortality rate was 23 % and it was highest in the last 3 months of pregnancy (Harris 1919).

During the influenza epidemic of 1957, pregnant women accounted for nearly half of the deaths of women of childbearing age. All deaths were attributed to respiratory insufficiency secondary to pulmonary edema and pneumonia (Greenberg et al. 1958). Mullooly et al. (1986) reviewed influenza complicating pregnancy from 1975 to 1979. There were four epidemics during

that 5-year period. Pregnant women sought outpatient medical attention for acute respiratory disease during the influenza season significantly more often than non-pregnant women. However, unlike the previously reported epidemics, there were no maternal deaths attributable to influenza, and the hospitalization rate was low at 2 per 1,000 (Mullooly et al. 1986).

During the last pandemic caused by the influenza A(H1N1) pdm09 virus in 2009, using a passive surveillance system in the USA, it was found that pregnant women were 7.2 times more likely to be hospitalized and 4.3 times more likely to be admitted to an intensive care unit (ICA) than non-pregnant women (Creanga et al. 2010). Among all deaths from influenza that year, 4.3 % were reported in pregnant women. This risk was higher if they had an underlying medical condition, were older, or were infected in the third trimester. Severe illness occurred in all pregnancy trimesters, but most cases (55 %) occurred in the third trimester. In a study by Neuzil et al. (1998), women in the third trimester were three to four times more likely to be hospitalized for an acute cardiopulmonary illness during influenza season than postpartum women. Asthma in pregnant women increased the rate of hospitalizations for a respiratory illness tenfold during influenza season. Bogers et al. (2011) also investigated the pregnancy outcomes and complications in all hospitalized pregnant women infected in 2009 by influenza A/H1N1 in the Netherlands. Most women were admitted from 28 weeks of gestation onward, which could have been due to the concern about the fetal condition, but more likely resulted from decreased pulmonary capacity caused by diaphragmatic elevation and decreased chest wall compliance. Pre-term birth was a recognized complication of the 2009 influenza A/H1N1 infection (Bogers et al. 2011). The perinatal outcomes of the 2009 influenza A/H1N1 in the United Kingdom were reported by Pierce et al. (2011). The authors found 10 deaths among 256 infants and increased risk of perinatal mortality in the infected women compared with the uninfected ones.

The effect of the influenza infection on the fetus is not clear. However there is an increased risk of spontaneous abortion, still birth, or prematurity. There is a distinct lack of prospective data on the effects of intrapartum, laboratory-confirmed influenza on the fetal outcome. Influenza has been associated with limb reduction and neural tube defects, including anencephaly (Lynberg et al. 1994; Aro et al. 1984; Coffey and Jessop 1959), although anencephaly has not been uniformly confirmed (Saxen et al. 1990). Irving et al. (2000) found no significant difference in the incidence of congenital malformations between women who had serum-confirmed influenza and controls. Widelock et al. (1963) studied the influenza epidemics between 1957 and 1960. They found neither the increased incidence of fetal death nor malformations in pregnant women who had influenza. In contrast, some studies unraveled the increased incidence of schizophrenia in people who were born 2–3 months after the influenza epidemic, which implies that maternal exposure to influenza in the second trimester, when fetal neurons are migrating, is a risk factor (Sham et al. 1992; Mednick et al. 1988). There have also been reports of an increased incidence of cleft lip (Leck 1963, 1969). Unfortunately, many studies are limited by recall and selection bias, making it unclear if there truly is a link. However, a direct teratogenic effect of influenza viruses appears unlikely and it is presumed that the fetus is affected by the infection indirectly through fever. In a study by Acs et al. (2005) the risk of congenital anomalies was reduced by the use of anti-fever drugs.

It should be highlighted that influenza is a clinical problem not just for pregnant women but also for the infant care due to their young age precluding vaccination against influenza. Mothers (or households in general) may be a source of influenza viruses for unprotected infants. Data from the 2003/2004 epidemic season in the United States indicated that the death rate from influenza in infants aged 0–6 months was 88/100,000 and only one third of the deaths occurred in children affected by chronic diseases,

such as bronchial asthma, chronic lung disease, or cardiovascular conditions (Glass et al. 2009).

2.2 Safety of Influenza Vaccination in Pregnant Women

Inactivated split or subunit influenza vaccines have been given to millions of pregnant women around the world with no harmful effects either for the mother or the child. The incidence of adverse reactions was similar among vaccinated and unvaccinated women and there was no increased risk of complications during pregnancy or a higher number of cesarean deliveries in vaccinated women.

The Centers for Disease Control and Prevention (CDC), in collaboration with the Food and Drug Administration (FDA), conducted a search of reports in the Vaccine Adverse Event Reporting System (VAERS) for pregnant women who received seasonal influenza vaccines from 1990 to 2009 to address the potential vaccine safety concerns. The study concluded that no unusual patterns of pregnancy complications or adverse fetal outcomes were observed in the VAERS reports on pregnant women after being given the influenza vaccine. The CDC is also conducting studies on flu vaccine safety and pregnancy through the Vaccine Safety Datalink (VSD). The study's findings provide reassurance that the flu vaccine given to pregnant women during the first trimester of pregnancy does not increase the risk of spontaneous abortion (CDC 2013a).

A matched case-control study of 252 pregnant women who received TIV in the 6 months before delivery determined no adverse events after vaccination among pregnant women and no difference in pregnancy outcomes compared with 826 pregnant women who were not vaccinated (Munoz et al. 2005).

During the 2000–2003 seasons, an estimated 2 million pregnant women were immunized and only 20 adverse events among women who received TIV were reported to VAERS, including nine injection-site reactions and eight systemic reactions (e.g. fever, headache and myalgias). In addition, three miscarriages were reported but these were not known to be causally related to the vaccination (Iscander et al. 2006). The rate of adverse events associated with TIV was similar to the rate of adverse events among pregnant women who received the pneumococcal polysaccharide vaccine in one small randomized controlled trial in Bangladesh and no severe adverse events were reported in any study group (Zaman et al. 2008). A recent international review of data on the safety of TIV concluded that no evidence exists to suggest harm to the fetus (Mak et al. 2008). There was no increased risk of cesarean section in vaccinated women and no increased risk of complications during pregnancy (Zaman et al. 2008; Munoz et al. 2005; Black et al. 2004).

2.3 Effectiveness of the Influenza Vaccination in Pregnant Women

Pregnant women have protective levels of specific anti-influenza antibodies after immunization (Munoz et al. 2005; Sumaya and Gibbs 1979). The passive transfer of anti-influenza antibodies that might pass the protection from vaccinated women on neonates has also been reported (Steinhoff et al. 2010; Englund et al. 1993; Sumaya and Gibbs 1979). The degree and duration of protection is directly dependent on the mother's influenza antibody titers and placental transfer efficacy, essentially defined by the time elapsed between immunization and delivery. The duration of passively acquired antibodies in infants depends on the initial cord concentration and is probably less than 6 months (Irving et al. 2000). Puck et al. (1980) reported a correlation between the level of cord blood influenza antibodies and the time of culture-documented influenza infection, showing that infants with high levels of influenza antibodies saw a delay in influenza infection.

Poehling et al. (2011) reported that hospitalized infants whose mothers received influenza vaccine during pregnancy were 45–48 % less likely to have laboratory-confirmed influenza during their first influenza season compared with infants of unvaccinated mothers. A prospective observational study among native Americans in

2002–2005 found that infants of immunized mothers had a 41 % decrease in the risk of laboratory-confirmed influenza infection and a 39 % reduction in the risk of hospitalization for an influenza-like illness (Eick et al. 2011).

One randomized controlled trial conducted in Bangladesh in which a flu vaccination was offered to pregnant women during the third trimester demonstrated a 29 % reduction in respiratory illness with fever among the mothers and a 36 % reduction among their infants during the first 6 months of life. In addition, infants born to vaccinated women had a 63 % reduction in laboratory-confirmed influenza illness during the first 6 months of life (Zaman et al. 2008).

Thompson et al. (2014) provided further substantial evidence on the effectiveness of the actual vaccination against influenza in pregnant women. Their study was based on a modern model of case-control study (so-called 'test negative design'), which minimized systematic errors (selection errors) and therefore had a significant impact on the reliability of the results obtained. The results of the study, demonstrating that influenza vaccination in pregnant women reduced the risk of contracting influenza by 50 %, should be considered noteworthy, contributing to improved influenza vaccination in pregnant women.

2.4 Current Recommendations Regarding the Influenza Vaccination in Pregnant Women

In the United States and Canada recommendations for the influenza vaccination in pregnant women in the second and third trimester of pregnancy with the use of inactivated vaccines have been around for more than a decade. They were first published by the CDC in 1997, in 2004 these recommendations were expanded, recommending administration of the influenza vaccination not only in the second and third trimesters, but also in the first trimester of pregnancy (in both healthy women and those affected by chronic diseases which constitute a risk factor for the severe and complicated course of influenza) (CDC 2005). In 2005, the WHO recommended vaccination for all pregnant women in the epidemic season (WHO 2005). Vaccination against influenza in pregnant women has also been also recommended by the American College of Obstetricians and Gynecologists (ACOG 2004). Pregnancy-related influenza vaccination recommendations were published during the 2012/2013 season in 23 European Union member states, with 13 countries recommending vaccination in any trimester and 10 countries recommending vaccination in the second or third trimester (ECDC 2013). Vaccination against influenza in pregnant women and postpartum women may be considered a way of protecting not only the mother but also the fetus, newborn and young infant (cocoon vaccination strategy). The only exception is the live attenuated vaccine administered intranasally, which is not recommended for use in pregnant women.

Despite official recommendations from experts, the level of vaccinations against influenza in the population of pregnant women is very low and varies from 2 to 20 % (CDC 2012). The low rate may stem from factors like: lack of physicians' recommendation, unavailability of vaccine in gynecological–obstetric surgeries, insufficient knowledge about influenza and its complications in pregnant women, insufficient knowledge about the safety and efficacy of the influenza vaccination in pregnant women among medical staff, insufficient knowledge of current recommendations for the influenza vaccination in pregnant women, the common belief among patients that the flu is not a serious disease, the misconception that vaccination against influenza can cause the flu, no reimbursement for the influenza vaccination by insurers. Unfortunately, the results of published studies indicate insufficient knowledge among medical staff regarding influenza vaccination in pregnancy: 40 % of the surveyed doctors and nurses who deal with pregnant women did not know that such women are in a group at higher risk of the complicated and severe course of influenza. Only 65 % of the staff were aware of the recommendations regarding influenza vaccinations for that group of patients (Yudin et al. 2009; Panda et al. 2011; Tong et al. 2011). Therefore, it must be concluded

that intensive educational efforts should be directed at medical staff, especially those taking care of women of childbearing age, to improve the immunization rate against influenza in this patient population.

3 Pertussis

3.1 Epidemiology and Clinical Course of Pertussis in Pregnant Women and Infants

Pertussis is caused by *Bordetella pertussis* and is a highly contagious disease as illustrated by secondary transmission rates as high as 90 % among susceptible household contacts. Despite the availability of effective pertussis vaccines for more than 40 years, pertussis is still endemic in many countries. While developing countries may account for up to 90 % of global reported cases, pertussis remains a public health issue in many developed countries, with high childhood vaccination coverage, which experience a change in the epidemiology characterized by a shift of the incidence over the last decade to older age groups often associated with a resurgence of infantile pertussis (Hewlett and Edwards 2005). However, the incidence of pertussis among pregnant and non-pregnant women remains similar and the infection during pregnancy has not been shown to result in enhanced morbidity (Matlow et al. 2013). In limited case reports, no pertussis-related deaths were reported in pregnant women. Rare reports of fetal morbidity from mothers with pertussis include one case of extradural hematoma (Bonnefoy et al. 2005) and another of laryngotracheal obstruction (Haugen et al. 2000), which apparently have not been related to the mother's pertussis infection.

In the pre-vaccination era, the majority of pertussis cases occurred in children who also represented the major source of transmission. As adults, their immunity was regularly boosted by recurrent exposure in the population. Protection was then passed from mothers on their infants through the placental transfer of antibodies. As maternal antibodies waned, infants became vulnerable to infection. This pattern is still observed in developing countries where not all children are adequately immunized during infancy (Hewlett and Edwards 2005; Rothstein and Edwards 2005).

After the use of pertussis vaccine had been established, the newly immunized pediatric group became protected. As a result of the widespread vaccine use, the circulation of *B. pertussis* within the community has been reduced and adolescents and adults are less regularly boosted by natural infection. Therefore, an increasing proportion of cases occur in adolescents and adults, who lost their vaccine-induced immunity (waning after the age of 4–12 years) and in infants, who receive fewer passive antibodies than infants did in the pre-vaccination era and who are too young to be immunized (Hewlett and Edwards 2005; Rothstein and Edwards 2005).

Household contacts, especially mothers with older siblings, are responsible for up to 75 % of *B. pertussis* infections in infants (Wendelboe et al. 2007). Mothers with pertussis at the time of delivery have a high chance of infecting newborns. Nooitgedagt et al. (2009) found serological evidence of current or recent pertussis in 2.5 % and 3.8 % of pregnant women, respectively. The authors suggested an efficient placental transfer of existing maternal IgG pertactine (PT) antibodies in pregnant women. There is evidence that maternal antibodies offer protection against pertussis in neonates (Healy et al. 2004) and low IgG–PT levels correlate with increased susceptibility to pertussis (Storsaeter et al. 2003). However, depending on the time of the onset of the mother's infection, the neonate may not be protected by maternal antibodies acquired through placental transfer.

It must also be emphasized that unvaccinated or incompletely vaccinated infants aged under 6 months have the highest risk of severe and life-threatening complications and death. Recent outbreaks of pertussis in Costa Rica (Ulloa-Gutierrez and Avila-Aguero 2008), California (Roehr 2010), and Saskatchewan (Lawrence 2010) have also been linked with increased reports of infant deaths.

In 2012, the CDC received more than 41,000 reports of pertussis infection in the US with 18 deaths reported, most of them in unvaccinated infants younger than 3 months (CDC 2013b). In Canada, approximately 2,500 cases were reported in 2012 with one fatality in a 1-month old infant (Alphonso 2012). These epidemiological data support the need for immunization against pertussis in pregnant women and women of childbearing age.

3.2 Safety of Pertussis Vaccination in Pregnant Women

The FDA classifies the Tdap vaccine as a pregnancy category C drug because there is insufficient evidence on the safety of its administration during pregnancy. Data are also insufficient on concerns about blunting the infant's immune response with the combined diphtheria, tetanus toxoid, and acellular pertussis (DTaP) vaccines. Nevertheless, the ACIP concluded that vaccination of pregnant women (who had not previously received Tdap) late in the second trimester or third trimester is an acceptable risk for both mother and fetus (CDC 2013b).

The safety of the Tdap vaccine during pregnancy is quite well established. Data from the Vaccine Adverse Event Reporting System, Sanofi Pasteur, and GlaxoSmithKline pregnancy registries, along with minor studies, have not suggest any increased frequency or unusual patterns of adverse events in pregnant women who received Tdap. However, the ACIP's conclusion is that administration of Tdap after 20 weeks of gestation is preferable in order to minimize the risk of uncommon adverse events and the possibility that any spurious association between Tdap-related adverse events and another illness might appear causative (ACIP 2013).

Zheteyeva et al. (2012) did not identify any consistent patterns in maternal, infant, or fetal outcomes after administration of the Tdap vaccine in pregnant women. The authors searched the VVAERS for the years 2000–2005 and identified 132 reports of Tdap administered to pregnant women There were no adverse events found in 55 (42 %) of the reports, no maternal or infant deaths were reported. The most frequent pregnancy-specific adverse event was spontaneous abortion in 22 (16.7 %) reports, injection-site reactions were the most frequent non-pregnancy–specific effect in 6 (4.5 %) reports, and one report identified a major congenital anomaly (gastroschisis).

The ACIP (2013) concluded that experience with tetanus-toxoid containing vaccines suggests no excess risk of severe adverse events for women receiving Tdap with every pregnancy. However, there is a need for safety studies on adverse events when Tdap is given during subsequent pregnancies.

3.3 Effectiveness of Pertussis Vaccination in Pregnant Women

Maternal pertussis immunization with Tdap vaccines seems more effective than postpartum vaccination of infant's close contacts. The cocoon strategy of pertussis immunization has been recommended in France and Germany, but there has been little compliance with the recommendation (Matlow et al. 2013). In Costa Rica, where the strategy was more aggressively implemented, infant deaths from pertussis decreased but it was unclear whether that was due to the effect of the immunization strategy or the natural waning of the outbreak (Ulloa-Gutierrez and Avila-Aguero 2008). A pilot project in Houston to immunize postpartum women before they left hospital was successful but was very resource-consuming (Healy et al. 2011).

For the 2011 ACIP recommendations, a decision analysis model was developed to assess the impact and cost effectiveness of the Tdap vaccination during pregnancy compared with vaccination immediately postpartum. The model showed that Tdap vaccination during pregnancy would prevent more infant cases, hospitalizations, and deaths compared with the postpartum dose. Based on the mathematical model, the Tdap vaccination during pregnancy might prevent 906 (range: 595–1,418) infant cases, 462 (range: 261–736) hospitalizations, and nine (range: 4–17)

deaths; a postpartum dose might prevent 549 (range: 361–860) infant cases, 219 (range: 124–349) hospitalizations and three (range: 1–6) deaths (ACIP 2013).

In a 2011 study, newborns whose mothers received Tdap during pregnancy were significantly more likely to have protective antibodies against pertussis than newborns whose mothers did not receive Tdap during pregnancy (Gall et al. 2011).

3.4 Current Recommendations Regarding Pertussis Vaccination in Pregnant Women

In January of 2013, the ACIP (2013) released a revised adult immunization schedule and recommended administering a dose of tetanus toxoid, reduced diphtheria toxoid, and acellular pertussis vaccine (Tdap) to all women during each pregnancy, regardless of their immunization history and stating that the optimal time for Tdap administration is between 27 and 36 weeks of gestation, although Tdap may be given at any time during pregnancy. It is worth noting that after receiving Tdap, a minimum of 2 weeks is required for a maximum immune response to the vaccine's antigens (Halperin et al. 2011; Kirkland et al. 2009) and the active transport of maternal immunoglobulin G does not take place in great quantities before the 30th week of gestation (Englund 2007). Additionally, new data indicate that maternal antipertussis antibodies are short-lived; therefore, the Tdap vaccination in one pregnancy will not provide high levels of antibodies to protect newborns during subsequent pregnancies (Healy et al. 2013). That is a change from the 2008 and 2011 recommendations, which advised that only women who had never received Tdap or those for whom it was 10 or more years since the previous boosters should get a dose of Tdap during pregnancy (ACIP 2008, 2011). Both sets of recommendations advised that a dose of Tdap be administered immediately after delivery if a woman did not received the vaccine during pregnancy. The ACOG Committee on Obstetric Practice supports the 2013 revised ACIP recommendations on the use of the Tdap vaccine during pregnancy (ACOG 2013).

The importance of vaccinating during each pregnancy is emphasized by the case of a 40-day old baby who died from pertussis; the baby's mother had received a postpartum Tdap dose 2 years earlier, but she developed a coughing illness 1 week before delivery (Matlow et al. 2013). Since the 'cocoon' method of vaccinating parents and all close contacts immediately after delivery has been shown ineffective and resource-heavy, Tdap vaccination for pregnant women might be a more feasible option for reducing pertussis transmission to unvaccinated newborns (ACIP 2012; Matlow et al. 2013).

The recommendation to vaccinate during each pregnancy is based on considerations of high pertussis rates, low vaccination rates in pregnant women, and hesitancy among health care providers to vaccinate when the mother's Tdap history is unknown (Matlow et al. 2013). Since the 2011 ACIP vaccination recommendations, the uptake of Tdap among pregnant women remains low. A survey of 1,231 women estimated that only 2.6 % of them received Tdap during their last pregnancy (ACIP 2013). The pertussis vaccination coverage rate among Korean women is even lower, amounting to 0.8 % (Seon et al. 2013). A low Tdap vaccine coverage among pregnant women is a result of lack of awareness among both patients and medical professionals. It should be pointed out that 80 % of patients would agree to the pertussis vaccination during pregnancy if that were recommended by an obstetrician-gynecologist (Varan et al. 2014), which underscores the role of medical professionals in the promotion of vaccinations.

4 Conclusions

Immunization during pregnancy is a well-established method for providing protection for both the mother and the newborn infant. Maternal vaccinations with Tdap and trivalent inactivated influenza vaccines have been shown

to be immunogenic and safe. Promoting patients' awareness about pertussis and influenza diseases and vaccine effectiveness, and encouraging the medical staff to recommend vaccination ought to increase the rate of vaccinated pregnant women and to reduce the risk of infections.

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

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Aerosolized GLP-1 for Treatment of Diabetes Mellitus and Irritable Bowel Syndrome

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Abstract

Diabetes is a global burden and the prevalence of the disease, in particular diabetes mellitus type 2 is rapidly increasing worldwide. After introduction of insulin into clinical therapy about 90 years ago a major number of pharmaceuticals has been developed for treatment of diabetes mellitus type 2. One of these, the incretin glucagon-like peptide 1 (GLP-1), like insulin, needs subcutaneous administration causing inconvenience to patients. However, administration of GLP-1 plays also a role for treatment of irritable bowel syndrome (IBS). To improve patient convenience inhaled insulin (Exubera®) was developed and approved but failed market acceptance some years ago. Recently, another inhalative insulin (Afrezza®) received market approval and GLP-1 may serve as another candidate drug for inhalative administration. This review analyzes the current literature investigating alternative administration of GLP-1 and GLP-1 analogs focusing on inhalation. Several formulations for inhalative administration of GLP-1 and analogs were investigated in animal studies, whereas there are only few clinical data. However, feasibility of GLP-1 inhalation has been shown and should be further investigated as such type of drug administration may serve for improvement of therapy in patients with diabetes mellitus or irritable bowel syndrome.

Keywords

Aerosol • Diabetes mellitus • Glucagon-like peptide 1 • Inhalation • Insulin • Irritable bowel syndrome

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1 Introduction

Diabetes mellitus is a global disease with increasing prevalence. Whereas diabetes mellitus type 1 is generally thought to be a an immune-associated disease resulting in a destruction of

insulin producing pancreatic β-cells and consecutive lack of insulin production, the much more frequent diabetes mellitus type 2 is caused by a major number of factors, namely obesity, sedentary lifestyle, westernized diet, and lack of physically activity primary resulting in an insulin resistance. Both types of diabetes differ strongly in their geographic distribution and especially the frequency of diabetes mellitus type 2 has largely increased in the last few decades mainly in Asia, where more than 60 % of worldwide cases of diabetes is expected to come from in 2030. Worldwide diabetes affects at least 382 million people as of 2013. This number is expected to increase to 592 million people in 2035, two thirds of whom live in low or middle income countries. Diabetes was responsible for 10.8 % of health expenditures (at least $548 billion) in 2013; expected to hit $678 billion in 2035, which demonstrates the global relevance and the requirement of efficient treatment (Abdullah et al. 2014; Atkinson et al. 2014; International Diabetes Federation 2013; Hu 2011).

Diabetes mellitus was first described more than 2,500 years ago in the Ebers papyrus and first successfully treated by injection of insulin in 1922 to Leonard Thompson a 14-year old boy who survived some more years under insulin therapy (Yaturu 2013; Quianzon and Cheikh 2012a; Rosenfeld 2002). At the beginning, insulin was exclusively administered by intramuscular injection, but for avoidance of patient traumatization subcutaneous application was rapidly established. Other modes of treatment, e. g. transdermal, ocular, oral, buccal, nasal, rectal, vaginal and transuterine were also investigated and some of them are still under development (Grant and Leone-Bay 2012; Siekmeier and Scheuch 2008a; Cefalu 2004; Belmin and Valensi 2003; Owens 2002). In the next decades a large number of compounds for mostly oral treatment of diabetes mellitus type 2 were developed and introduced into the market (e. g. biguanides (beginning between 1920 and 1930), sulfonylureas (beginning in the 1950s), thiazolidinediones (beginning in the 1990s), meglitinides (beginning in the 1990s), α-glucosidase inhibitors (beginning in the 1990s), glucagon-like peptide 1 receptor agonists (GLP-1 receptor agonists) and dipeptidyl peptidase 4 inhibitors (DPP-4 inhibitors, beginning in the 2000s), amylin agonists (beginning in the 2000s) and sodium glucose co-transporter 2 inhibitors (SGLT-2 inhibitors, beginning in the 2010s)) (Chao 2014; Rosenwasser et al. 2013; Quianzon and Cheikh 2012b).

Glucagon-like peptide 1 (GLP-1) is an incretin hormone mostly made in enteroendocrine L cells of distal ileum and colon but also in the duodenum and jejunum. GLP-1 rapidly increases in plasma within minutes of eating a carbohydrate- or fat-containing meal. Physiologically, GLP-1 stimulates synthesis of insulin, suppresses glucagon secretion, and delays gastric emptying (Quianzon and Cheikh 2012b; Drucker 2006; Drucker and Nauck 2006). Beyond this, GLP-1 enhances pancreatic β-cell proliferation and promotes expansion of islet mass, elicits a potent aversive effect contributing to its anorectic effect and improves myocardial function and output. Bioactive GLP-1 is generated from GLP-1(1–37) and exists as two equipotent circulating molecular forms, GLP-1(7–37) and GLP-1(7–36)amide representing the majority of active GLP-1 circulating in human plasma. The molecule is rapidly inactivated by DPP-4 with a half-life time of 1–2 min only. Exenatide and liraglutide were the first GLP-1 agonists introduced into the market, whereas others (lixisenatide, albiglutide, dulaglutide and taspoglutide) were introduced later on or are in the approval process. GLP-1 agonists are administered in patients with diabetes mellitus type 2 by means of subcutaneous injection and beyond lowering blood glucose and plasma HbA_{1c} may produce satiety and weight loss but also nausea and vomiting (Quianzon and Cheikh 2012b; Drucker 2006; Drucker and Nauck 2006). Proteolytic cleavage of endogenous GLP-1 or administered GLP-1 agonists can be inhibited by DPP-4 inhibitors (i. e. sitagliptin, saxagliptin, linagliptin, alogliptin, and vildagliptin), which in contrast to GLP-1 agonists can be administered orally and are well tolerated. However, DPP-4 inhibitors can be also given alone or combined with other antidiabetic compounds (e.g., metformin) (Nauck and Vardarli 2010). Beyond the described use of GLP-1 and its analogs for

treatment of diabetes mellitus clinical studies have also demonstrated a reduction of acute exacerbations, a relaxation of gut musculature and pain relief after subcutaneous administration of ROSE-010, a GLP-1 analog, in patients with irritable bowel syndrome (IBS) (Camilleri et al. 2012; Hellström et al. 2009, 2012).

2 Aerosol Treatment of Diabetes

First studies on insulin inhalation were published by independent groups in 1924 and 1925, i. e. only 2 years after the start of the therapeutic insulin era (Rosenfeld 2002; Gänsslen 1925; Heubner et al. 1924; Laqueur and Grevenstuk 1924). However, it required about 80 more years until the first inhalative insulin (Exubera®, (Nektar Therapeutics/Pfizer) received market approval by FDA and EMA in 2006 for patients with diabetes mellitus types 1 and 2 (Siekmeier and Scheuch 2008a; Cefalu 2004; Owens 2002). Unfortunately, due to missing reimbursement by public health insurance companies in major markets (after recommendations of IQWIG and NICE in Germany and Great Britain), bulky devices with tedious handling, concerns of lung safety and limited exposure in individuals without pulmonary disease requiring lung function controls, there was no widespread use of the device and after about 1 year Pfizer took off Exubera® from the market. After stop of marketing of Exubera® most other companies stopped further development of projects on inhalative insulin. Mannkind Pharmaceuticals just received regulatory approval of their product (Afrezza®) for treatment of diabetes mellitus types 1 and 2 by means whereas Aradigm is still waiting for cooperation partners (Aradigm 2014; Mannkind Pharmaceuticals 2014; Hickey 2013). Afrezza® is based on Technosphere® technology, a versatile drug delivery platform enabling pulmonary delivery of even other pharmaceuticals than insulin, e.g. GLP-1 and calcitonin (Grant and Leone-Bay 2012; Cassidy et al. 2011; Marino et al. 2010; Potocka et al. 2010).

As described for insulin before, alternative routes, e. g. oral, nasal and inhalative routes for administration of GLP-1 agonists are also under intensive study in order to improve patient's inconvenience to administration, local pain and irritation during injection (Wang et al. 2013, 2014; Zhang et al. 2014; Ahn et al. 2013; Steinert et al. 2009, 2010; Jin et al. 2009; Beglinger et al. 2008; Gedulin et al. 2008). Focusing on pulmonary delivery we performed a Pubmed search for GLP-1 and analogs approved or under study (GLP-1, exenatide, liraglutide, lixisenatide, albiglutide, dulaglutide, taspoglutide, ROSE-010) and the terms aerosol or inhalation. However, only a small number of publications were found which is described in detail below.

3 *In Vitro* Studies and Animal Studies Investigating the Inhalation of GLP-1

In the first study published in the field of GLP-1 inhalation, Gedulin et al. (2008) investigated pharmacokinetics and biological activity of exenatide after delivery to different epithelial surfaces of the intestinal and respiratory tracts in Harlan-Sprague-Dawley rats and diabetic db/db mice. The authors studied the effect of intraduodenal delivery (administration of 1 mg/250 μg saline in rats 1.5 h after completion of surgery), sublingual delivery (administration of 5 μl containing 210 μg in rats and 3 μl containing 100 μg in mice), intranasal delivery (administration of 100 μg in 3 μl saline into one nostril in rats), intratracheal delivery (administration of 30 μl saline with or without exenatide in rats and 20 μl saline with or without 1 μg exenatide in mice), pulmonary delivery (rats and mice exposed 10 min to aerosolized exenatide (2 mg in 2 ml nebulized in a rate of 0.2 mg/min in an air flow of 5 l/min) in a 4.5 l chamber), intravenous delivery and subcutaneous injection. Parameters under study were plasma concentrations of glucose, insulin and exenatide. All deliveries of exanatide were followed by peak plasma concentrations within 30 min after administration. Beside differences in the kinetics of absorption and elimination, large differences were observed for the area under the plasma concentration *vs.* time curves

up to 480 min (AUC_{480}), the maximum plasma concentrations $C_{max.}$ and in the relative bioavailability depending on the dose and mode of administration. Intraduodenal (1,000 µg) and sublingual administration (210 µg) resulted in values of AUC_{480}, $C_{max.}$ and relative bioavailabilities of 2,895 pM/min, 0.115 pM/µg, and 0.0053 %, and 42,747 pM/min, 2.11 pM/µg and 0.37 %, respectively. The corresponding values for administration into the respiratory tract were 92,100 pM/min, 16.06 pM/µg and 1.68 % for intranasal (100 µg), and 100,285 pM/min, 0.71 pM/µg and 0.092 % (considering the wastage within the chamber 8–9 %) for aerosol (2,000 µg), and 1,556,000 pM/min, 327.1 pM/µg, and 13.6 % for intratracheal delivery. However, the highest values were observed after intravenous and subcutaneous administration of 210 µg (11,482,000 pM/min, 3,757 pM/µg and 100 %, and 7,080,240 pM/min, 134.3 pM/µg and 61.7 %, respectively). In another experiment, doses of 2, 21, and 210 µg were administered intratracheally in rats and pharmacokinetic parameters were compared with subcutaneous and intravenous administration of exenatide. The maximum plasma concentrations were dose-dependent (58 pM, 667 pM, and 25 nM, respectively). Peak plasma concentrations ($t_{max.}$) were obtained 20–30 min after delivery and 61–74 % of peak plasma concentrations were observed within 5 min after dosing. The corresponding values of relative bioavailability compared with intravenous administration were 7.3 %, 5.3 %, and 14.8 %, respectively. In db/db mice, sublingual administration of 100 µg exenatide was followed by a mean plasma concentration of 1,740 pM 1 h after dosing and a significant reduction of plasma glucose concentrations, whereas intratracheal administration of 1 µg resulted in a plasma concentration of 186 pM 4.5 h after administration and a significant reduction of plasma glucose concentration. Even after 10 min aerosol exposure, exenatide was detectable 1 h after later accompanied by a significant reduction of plasma glucose and a fourfold elevation of plasma insulin concentration. In summary, the authors demonstrated that the respiratory tract may serve as an interesting target for non-invasive delivery of GLP-1 for treatment of diabetes mellitus (Gedulin et al. 2008) (Table 1).

The feasibility of inhalation of native GLP-1 by means of the Technosphere® platform was demonstrated in rats by Leone-Bay et al. (2009). These authors prepared GLP-1 Technosphere® powders containing 5 %, 10 % and 15 % GLP-1. Particles were characterized by means of scanning electron microscopy (SEM), Andersen cascade impactor, and high performance liquid chromatography (HPLC) for surface morphology, particle size, and GLP-1 content, respectively. Doses of 0.12 mg, 0.17 mg, and 0.36 mg GLP-1 were intrapulmonary administered to the lungs of Sprague-Dawley rats by means of an insufflation device and instillation of 0.125 mg GLP-1 (0.1 ml liquid) served for reference. Plasma concentrations of GLP-1 and FDKP (bis-3,6(4-fumarylaminobutyl)-2,5-diketopiperazine and polysorbate 80) were determined up to 60 min after dosing. The effect of 0.3 mg GLP-1 (particle load 15 % GLP-1) once daily on food consumption was determined in comparison with air, serving as control in another experiment in the same animal model. Food consumption and body weight were parameters under study for up to 4 days. Obese Zucker diabetic fatty rats were used for the investigation of the effect of GLP-1 on glucose metabolism and endocrine pancreas. In one group, rats were treated with 0.3 mg GLP-1 (particle load 15 % GLP-1) once daily for up to 4 days, whereas air served as control in the other group. On day 4, an intraperitoneal glucose tolerance test was performed and blood was collected up to 90 min after dosing for determination of blood glucose, plasma GLP-1, and serum insulin. Furthermore, evaluation of β-cell proliferation, insulin expression, and apoptosis was performed by histological and immune histological means and by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The determination of particle size demonstrated respirable fractions (i. e. aerodynamic diameters between 0.5 and 5.8 µm) of 48.8 %, 57.0 %, and 32.2 % of the Technosphere® formulations (5 %, 10 %, and 15 % GLP-1, respectively). Rats treated with 5 %, 10 %, and 15 % GLP-1 Technosphere® powder showed a rapid increase of GLP-1 plasma concentration (maximum plasma concentrations $t_{max.}$ after 2 min, 10 min, and 10 min respectively) and dose-dependent peak plasma concentrations

Table 1 Brief characteristics of *in vivo* studies with inhalation of GLP-1 or GLP-1 analogues

Study	Drug	Platform	Animal	Mode of administration	Result
Gedulin et al. (2008)	Exenatide (Exendin-4)	Solution and aerosol	Sprague-Dawley rats, db/db mice	Intratracheally by means of liquid; pulmonary by an inhalation chamber; other modes of administration	Rapid absorption within 30 min after intratracheal delivery or aerosol inhalation; glucose lowering effect
Leone-Bay et al. (2009)	GLP-1	Technosphere® powder (FDKP)	Sprague-Dawley rats, obese Zucker diabetic fatty rats	Intrapulmonary by an insufflation device; liquid instillation	Rapid absorption of GLP-1 and FDKP within 10 min after delivery; decreased food consumption after GLP-1 delivery; lower glucose and higher insulin content after i.p. glucose tolerance test in Zucker rats; no differences in apoptotic index and proliferation of β-cells in Zucker rats
Qian et al. (2009)	GLP-1 agonist BMS-686117	Three powder formulations; I: 80/20 BMS-686117/trehalose, II: 100 % BMS-686117, III: 20/80 BMS-686117/mannitol	Sprague-Dawley rats	Intratracheally by a dry powder insufflator	Best properties *in vitro* and *in vivo* for powder formulation III; more rapid absorption after intratracheal than subcutaneous delivery
Kim et al. (2011a)	Exendin-4	Porous large PLGA microspheres adsorbed with Ex4-C_{16} or Ex4	ICR mice, db/db mice	Pulmonary by an insufflator device and an air pump	Efficient deposition of microspheres throughout the lungs; no lung toxicity; longer hypoglycemic effect of Ex4-C_{16} loaded PLGA microspheres than of Ex4 loaded PLGA microspheres
Kim et al. (2011b)	Exendin-4	Albumin-coated porous hollow PLGA microparticles adsorbed with Ex4-C_{16} or Ex4	ICR mice, db/db mice	Intratracheally by an insufflator device and an air pump	Coated PLGA microparticles well deposited and totally dispersed throughout the lung without lung toxicity; longer hypoglycemic effect of Ex4-C_{16} than Ex4 loaded coated PLGA microparticles
Lee et al. (2012a)	Exendin-4	Deoxycholic acid-modified glycol chitosan (DOCA-GC) nanogels containing Ex4-C_{16} or Ex4	db/db mice	Intratracheally by a microsprayer aerolizer	Rapid deposition of DOCA-GC nanogels remaining in the lungs for about 72 h; moderate toxicity *in vitro* but not *in vivo*; longer hypoglycemia induced by Ex4-C_{16} DOCA-GC nanogels than by native Ex4-C_{16}

(continued)

Table 1 (continued)

Study	Drug	Platform	Animal	Mode of administration	Result
Lee et al. (2012b)	Exendin-4	Ex4-C_{16} and Ex4	ICR mice, db/db mice	Intratracheally insufflation	Ex4 more rapidly absorbed from the lungs than Ex4-C_{16}; stronger hypoglycemic effect of higher doses Ex4-C_{16} and Ex4; delayed nadir of blood glucose but stronger hypoglycemic effect of Ex4-C_{16} than of Ex4
Lee et al. (2013)	Exendin-4	Chitosan-coated PLGA nanoparticles loaded with Ex4 or Ex4-C_{16}	ICR mice, db/db mice	Intratracheally by insufflation	Ex4 more rapidly absorbed from the lungs than Ex4-C_{16}; delayed nadir of blood glucose but stronger hypoglycemic effect of Ex4-C_{16} than of Ex4
Hellström et al. (2012)	ROSE-010, exendin-4	Technosphere® powder (FDKP)	Sprague-Dawley rats	Pulmonary insufflation an air pump and an insufflation chamber	Subcutaneously administered GLP-1 and ROSE-010 increase small bowel migrating myoelectric complex (MMC) and inhibit myoelectric spiking activity; insufflation of ROSE-010 also followed by increased MMC and inhibited myoelectric spiking activity, i. e. inhibited gut motility
Marino et al. (2010)	GLP-1(7–36) amide (MKC253)	Technosphere® powder (FDKP)	Healthy humans, diabetic patients	Inhalation	Rapid increase and decline of GLP-1 plasma content after inhalation; lowering of plasma glucose and increase of insulin after inhalation

Ex4 Exendin-4, *Ex4-C_{16}* Palmitylated Ex4, *FDKP* Bis-3,6(4-fumarylaminobutyl)-2,5-diketopiperazine and polysorbate 80, *GLP-1* Glucagon-like peptide 1, *ICR* Imprinted control region, *PLGA* poly(lactic-co-glycolic acid)

($C_{max.}$) and areas under the concentration *vs.* time curve (AUC) (2,660 ± 1,840 pM, 4,990 ± 2,400 pM, 11,700 ± 729 pM and 57,100 ± 47,600 pM/min, 92,600 ± 50,700 pM/min, and 228,000 ± 52,500 pM/min, respectively). The corresponding half-life times ($t_{1/2}$) of circulating GLP-1 were 14.9 min, 9.5 min, and 10 min, respectively. However, values of $t_{max.}$, $C_{max.}$, AUC, and $t_{1/2}$ after pulmonary instillation of GLP-1 were 20 min, 281 ± 101 pM, 10,600 ± 3,840 pM/min, and 50 min, respectively. Plasma concentrations of FDKP were also determined after 5 %, 10 %, and 15 % GLP-1 Technosphere® powder. The maximum concentrations of FDKP were observed 10 min after dosing with average $C_{max.}$ values of 8.1 μg/ml, 4.8 μg/ml, and 7.1 μg/ml, respectively, demonstrating rapid degradation and absorption after pulmonary deposition. In another experiment, food consumption was determined in normal rats after administration of 2 mg 15 % GLP-1 Technosphere® powder *vs.* air control once daily

on 4 consecutive days up to 24 h after dosing. First dosing was followed by a reduction of food consumption (75 % at 1 h, 43 % at 6 h) persisting through successive doses. In another experiment, Zucker diabetic obese rats were treated with 2 mg 15 % GLP-1 Technosphere® powder or air in the control group once daily on 4 consecutive days and on day 4 of dosing, an intraperitoneal glucose tolerance test was performed. Parameters under study were plasma concentrations of GLP-1, glucose and insulin, and β-cell mass and apoptosis. Plasma concentrations of GLP-1 rapidly increased after administration ($t_{max.}$: 15 min, $C_{max.}$: 10,600 pM). Starting from baseline concentrations of about 325 mg/dl, there was a strong increase of plasma glucose concentrations in the untreated group which were significantly higher than in the GLP-1 treated group up to 90 min after dosing (704 ± 75 mg/dl *vs.* 484 ± 119 mg/dl). On the other hand, animals of the control group showed a significant decrease of insulin concentrations, whereas no change was observed in the GLP-1 treated group. Histological analyses demonstrated an increase of insulin-secreting cells per islet in the GLP-1 treated group *vs.* the controls (51.5 ± 10.1 % vs. 42.1 ± 8.4 %) whereas β-cell mass and apoptosis showed no differences suggesting that the reduced concentrations in plasma glucose observed in the intraperitoneal glucose tolerance test result from insulin induction but not alteration in proliferation or apoptosis. Taken together, the results suggest the feasibility of GLP-1 inhalative administration by means of the Technosphere® platform in rats (Leone-Bay et al. 2009) (Table 1).

In another early study Qian et al. (2009) investigated morphology and pharmacodynamics of the GLP-1 receptor agonist BMS-686117, which is an 11-mer with a molecular weight of 1,528.7 g/mol, low solubility between pH 4 and 6.5 in water (about 1 µg/ml or less) and no oral bioavailability. Morphology of lyophilized BMS-686117 powder and three spray-dried prototype powder formulations (I: 80/20 BMS-686117/trehalose, II: 100 % BMS-686117 and III: 20/80 BMS-686117/mannitol) was investigated by means of SEM. Lyophilized BMS-686117 exhibited the morphology of irregular flakes with a large size range of 2–100 µm which also had a poor flow characteristics whereas all prototype powder formulations had more homogenous particle sizes between 2–10 µm and better flow characteristics. However, the prototype powder formulation III (20/80 BMS-686117/mannitol) showed the narrowest particle size distribution (around 2–3 µm) and the most regular spherical appearance whereas prototype formulations I and II (80/20 BMS-686117/trehalose and 100 % BMS-686117) had size ranges <5 µm with a broader size distribution and a morphology of more collapsed, hollow, and irregular particles. Pharmacokinetic patterns were investigated in Sprague-Dawley rats for doses of 1 mg/kg of lyophilized powder and all three prototype formulations administered intratracheally by means of a dry powder insufflator and subcutaneously and intravenously administered doses of 0.08 mg/kg and 0.1 mg/kg, respectively. Intratracheal administration was followed by a more rapid absorption (time to reach $C_{max.}$, $t_{max.}$) and higher maximum plasma concentrations ($C_{max.}$) than subcutaneous (s.c.) administration (lyophilized powder, prototypes I, II, and II, and s.c.; $t_{max.}$: 0.3 h, 0.44 ± 0.13 h, 0.30 ± 0.17 h, 0.67 ± 0.29 h and 1.2 ± 0.5 h, respectively; $C_{max.}$: 55.9 ± 51 nM, 91.4 ± 14.1 nM, 152.8 ± 43.3 nM, 191.5 ± 48.1 nM and 11 ± 2 nM, respectively) and except that for lyophilized BMS-686117 powder the areas under the concentration *vs.* time curves ($AUC_{last.}$) were also higher after intratracheal administration (49.4 ± 55 nM/h, 103 ± 15 nM/h, 151 ± 51 nM/h, 299 ± 43 nM/h, and 55 ± 5 nM/h , respectively). All three powder prototypes showed plasma half-life times ($t_{1/2}$) in the same range of the injected peptide (0.84–1.4 h) and concentrations detected for at least 6 h. The highest absolute bioavailability was found for intratracheally administered prototype III formulation (2.5 ± 2.8 %, 5.6 ± 0.5 %, 7.7 ± 2.6 %, 15.3 ± 2.2 %, and 34.4 ± 3.1 %, respectively). For prototype III this corresponds to a bioavailability of 45 % relative to subcutaneous administration which together with a coefficient of variation of about 14 % indicates its potential therapeutic use. In summary, inhalation of the

spray-dried formulation of 20/80 BMS-686117/mannitol was shown to have a rapid onset of action, sufficient bioavailability and modest variability in vivo demonstrating **its potential therapeutic use** (Table 1).

Biophysical characteristics and pharmacological properties of porous large poly(lactic-co-glycolic acid) (PLGA) microspheres adsorbed with palmityl-acylated exendin-4 (Ex4-C_{16}) or native exendin-4 (Ex4) were studied by Kim et al. (2011a). Surface morphology and porosity of porous PLGA microspheres, adsorption of Ex4-C_{16} or Ex4 on PLGA microspheres and the release of Ex4-C_{16} or Ex4 from PLGA microspheres were determined by means of SEM, confocal laser scanning microscopy (CLSM) and the measurement of fluorescence release from labeled particles. Pulmonary deposition of porous PLGA microspheres was determined in imprinting control region (ICR) mice or type 2 diabetic C57BL/6 db/db mice up to 6 h after direct administration into the lungs *via* the trachea by means of an insufflator device and an air pump. Lung toxicology was studied in db/db mice by histological evaluation of lung tissues 2 weeks after administration. The hypoglycemic effects of porous PLGA microspheres and Ex4-C_{16}, or Ex4 loaded PLGA microspheres were determined in db/db mice for up to 120 h after administration. The results of the biophysical analysis demonstrated that the prepared microspheres had a particle size and a mass median aerodynamic diameter (MMAD) of 16.2 ± 2.3 μm and 4.4 ± 0.5 μm, which is in the appropriate size range for respiration and a pore size range of 0.5–2 μm. A loading amount of 5.0 ± 0.7 % was observed for Ex4-C_{16} on PLGA microspheres, which was significantly higher than that for Ex4. On the other hand, release of Ex4-C_{16} from PLGA microspheres was much slower than that observed for Ex4 (94.5 ± 3.2 % *vs.* 31.2 ± 4.2 % after 6 h, respectively). Using the optimized particles the authors reported an efficient deposition of PLGA microspheres throughout the lungs including the alveoli. Histological evaluation of lung tissues 2 weeks after administration showed no toxicity and no differences between untreated db/db mice and mice treated with blank porous PLGA microspheres or Ex4-C_{16} loaded PLGA microspheres. Also, in db/db mice a hypoglycemic effect was observed after pulmonary administration of Ex4-C_{16} or Ex4 loaded PLGA microspheres, compared with unloaded porous PLGA microspheres. Administration of Ex4-C_{16} loaded PLGA microspheres was followed by a strong decrease of blood glucose concentration from 466.4 ± 72.6 mg/dl to a bottom value of 155.6 ± 41.9 mg/dl ($p < 0.001$), which was also significantly lower than that of the control group (406.0 ± 67.9 mg/dl, $p < 0.005$). Even 5 days after administration, a lower concentration of blood glucose was observed in animals treated with Ex4-C_{16} loaded PLGA microspheres (290.3 ± 98.0 mg/dl), whereas no more relevant effect was observed in animals treated with Ex4 loaded PLGA microspheres 2 days after dosing. In summary, the results demonstrated the potential of porous large PLGA microspheres as a long-acting slow-release carrier for Ex4-C_{16} (Table 1).

In another study, Kim et al. (2011b) investigated biophysical characteristics and pharmacological properties of albumin-coated porous hollow PLGA microparticles adsorbed with palmityl-acylated exendin-4 (Ex4-C_{16}) or native exendin-4 (Ex4). Surface morphology and porosity of porous hollow PLGA microspheres, adsorption of Ex4-C_{16} on the coated microparticles and the release of Ex4-C_{16} from the coated PLGA microparticles were determined by means of SEM, CLSM, and the measurement of fluorescence release from labeled particles. Images of the aerosolization were made for both coated and non-coated PLGA microparticles with a digital camera after actuation. Pulmonary deposition of coated PLGA microparticles was determined in ICR mice up to 3 h after direct administration into the lungs *via* the trachea by means of an insufflator device and an air pump. Evaluation for cytotoxicity was performed *in vitro* in the models of the human lung adenocarcinoma epithelial cell lines Calu-3 and A549, which were incubated with coated or uncoated PLGA microparticles for up to 48 h, and *in vivo* 2 weeks after

administration of coated or uncoated PLGA microparticles. The hypoglycemic effects of coated PLGA microparticles and Ex4-C_{16} or Ex4 loaded coated PLGA microparticles were determined in db/db mice for up to 120 h after administration. Particle size and MMAD of the Ex4-C_{16} loaded coated PLGA microparticles were 17.2 ± 2.1 μm and 3.2 ± 0.3 μm, respectively, which is in the appropriate size range for respiration and the loads of albumin-coating and Ex4-C_{16} per mg particle were 220 ± 23 μg and about 38.1 μg, respectively. Imaging of aerosolization demonstrated a good mobility of aerosol particles and a 1.6-fold better aerosolization efficiency of coated *vs.* uncoated PLGA microparticles (3.3 ± 0.3 *vs.* 2.1 ± 0.5 mg, $p < 0.007$). Ex4-C_{16} was almost linearly released from coated PLGA microparticles without a marked initial burst (about 26 % after 1 day and about 90 % after 5 days). In ICR mice, the administered coated PLGA microparticles showed a good deposition and were totally dispersed throughout the lungs, including the alveoli, 3 h after administration. Analysis for cytotoxicity demonstrated a relatively low toxicity to both cell lines within 48 h which was slightly, but not significantly, lower for coated than for non-coated microparticles. However, histological evaluation of lung tissues performed in db/db mice 2 weeks after administration showed no toxicity and no differences between untreated mice and mice treated with coated PLGA microspheres or Ex4-C_{16} loaded coated PLGA microspheres. A strong and long lasting hypoglycemic effect was observed in db/db mice after administration of Ex4-C_{16} loaded coated PLGA microparticles compared with that of Ex4 loaded coated PLGA microparticles and unloaded coated PLGA microparticles serving as control. Administration of 120 μg of Ex4-C_{16} loaded coated PLGA microparticles was followed by a glucose concentration of 150.7 ± 60.8 mg/dl after about 8–12 h, which was significantly lower than that of the control (413.0 ± 81.5 mg/dl, $p < 0.001$) and the mean baseline value (445.5 ± 67.3 mg/dl, $p < 0.0001$). The hypoglycemic effect remained significant until 5 days after dosing (331.4 ± 61.8 mg/dl), whereas in mice treated with Ex4 loaded coated PLGA microparticles only a little effect was observed 2 days after administration when compared with the initial concentration (425.3 ± 64.0 mg *vs.* 485.3 ± 59.1 mg/dl, respectively). In summary, the study demonstrated that the albumin-coated porous hollow PLGA microparticles adsorbed with palmityl-acylated exendin-4 may serve as a long-time slow release delivery system (Table 1).

Lee et al. (2012a) investigated inhalable deoxycholic acid-modified glycol chitosan (DOCA-GC) nanogels which contained palmitoyl acylated exendin-4 or non-acylated exendin-4 (Ex4-C_{16} or Ex4, respectively). The particles were prepared by self-assembly and physicochemically characterized for size, surface morphology, and stability (release of Ex4-C_{16} or Ex4). Toxicity of the DOCA-GC nanogels was assayed *in vitro* in the models of human lung epithelial cells (A549 and Calu-3) and in the mice model. The spherical and compact particles with a diameter of about 220 nm were administered to type 2 diabetic C57BL/6 db/db mice intratracheally by means of a microsprayer aerolizer. Blood glucose concentrations were determined in animals treated with Ex4-C_{16} nanogel, Ex4 nanogel, Ex4-C_{16}, Ex4, and saline (for reference) up to 72 h after administration. Pulmonary deposition was monitored after administration of Cy5.5 labeled DOCA-GC nanogels up to 24 h (whole body images) and 72 h (excised lungs). The investigators observed a lower incorporation of Ex4-C_{16} compared with Ex4 into DOCA-GC nanogels (50.9 ± 7.8 % *vs.* 81.4 ± 4.9 %, respectively). However, the release of Ex4-C_{16} from nanogels was delayed when compared with Ex4 (release over 3 days *vs.* about 90 % release within 1 day, respectively). In mice, DOCA-GC nanogels were rapidly deposited and remained in the lungs for about 72 h. Strong differences were observed for intensity and duration of the hypoglycemic effect depending on the type of the administered compound. The hypoglycemic effect was weaker for Ex4-C_{16} DOCA-GC nanogels, compared with that for Ex4 DOCA-GC nanogels (glucose

concentration 175.3 ± 61.5 mg/dl *vs.* 135.8 ± 72.2 mg/dl, respectively) and the maximum effect was clearly delayed (12 h *vs.* 4 h, respectively). Accordingly, the duration of the hypoglycemic effect was longer for Ex4-C_{16} DOCA-GC nanogels than for Ex4 DOCA-GC nanogels (glucose concentration after 48 h; 273.0 ± 46.1 mg/dl *vs.* 413.5 ± 106.9 mg/dl, respectively). No difference of the hypoglycemic effect was found between Ex4 DOCA-GC nanogels and native Ex4. However, the hypoglycemia induced by Ex4-C_{16} DOCA-GC nanogels was greater than that induced by native Ex4-C_{16} (14,266 ± 2,859 mg/h/dl *vs.* 8,371 ± 1,318 mg/h/dl, respectively). A mild but acceptable cytotoxicity was observed at higher concentrations of DOCA-GC. Histology of lung tissue of mice treated with DOCA-GC nanogels 1 week after treatment showed no difference when compared with untreated controls. The authors conclude that Ex4-C_{16} loaded DOCA-GC nanogels can be used as a long-acting inhalation delivery system for treatment of diabetes mellitus type 2. However, potential lung toxicity, especially during long-term administration should be addressed (Table 1).

In another study Lee et al. (2012b) investigated particle size distribution of palmitic acid modified exendin-4 (Ex4-C_{16}), lung deposition, body distribution of Cy5.5 labeled Ex4-C_{16}, and non-modified Cy5.5 labeled exendin-4 (Ex4) in ICR mice, and the hypoglycemic effects of two different doses of Ex4-C_{16} and Ex4 in comparison with saline in type 2 diabetic C57BL/6 db/db mice after intratracheal insufflation by means of a micro-sprayer. Particle size analysis by means of a Zetasizer and a He-Ne laser beam at a concentration of 150 μg/ml in PBS pH 7.4 revealed a size of Ex4-C_{16} micelles of 228.8 nm. After insufflation of Cy5.5 labeled Ex4 or Ex4-C_{16} into the tracheal region, whole body images were obtained up to 3 h after administration of Ex4 and up to 8 h after administration of Ex4-C_{16}. Ex4 was rapidly absorbed from the lungs disappearing after 1–2 h and an increased kidney uptake was found up to 3 h after dosing. In contrast, Ex4-C_{16} remained about 4 h in the lungs with a small amount even visible after 8 h and no significant amounts were found after 8 h in the kidneys. The hypoglycemic effects of two different doses Ex4-C_{16} and Ex4 (75 and 150 nmol/kg) were determined in comparison with saline in type 2 diabetic C57BL/6 db/db mice up to 72 h after administration. Nadirs of blood glucose concentrations were observed 4.1 ± 0.3 h and 4.3 ± 1.1 h after administration of Ex4 and significantly later, 7.8 ± 2.2 and 8.2 ± 1.4 h after Ex4-C_{16} (doses of 75 nmol/kg and 150 nmol/kg, respectively). The nadirs were insignificantly lower for Ex4 than for Ex4-C_{16} (75 nmol/kg and 150 nmol/kg; Ex4: 92.8 ± 26.1 mg/dl and 81.9 ± 29.9 mg/dl, respectively; Ex4-C_{16}:115.7 ± 37.1 mg/dl and 91.8 ± 23.3 mg/dl, respectively). However, the times required for a rebound of blood glucose concentration to 150 mg/dl were longer for Ex4-C_{16} than for Ex4 (12.2 h *vs.* 4.1 h and 18.1 h vs 5.2 h for doses of 75 nmol/kg and 150 nmol/kg, respectively). The corresponding hypoglycemic effect was also higher after administration of Ex4-C_{16} than that after Ex4 (20.3 ± 5.0 % *vs.* 6.0 ± 2.6 %, $p < 0.02$ and 27.5 ± 6.2 % *vs.* 12.1 ± 4.4 %, $p < 0.02$, respectively). The data suggest the presence of significant hypoglycemic effects of intratracheally administered Ex4 and Ex4-C_{16}. However, due to delayed pulmonary absorption and albumin binding, inhalation of Ex4-C_{16} is followed by a later nadir of blood glucose concentration and a longer duration of drug action (Table 1).

The investigation of the characteristics of inhalable chitosan-coated PLGA nanoparticles containing palmitic acid-modified exendin-4 (Ex4-C_{16}) was a subject of another study by Lee et al. (2013). In brief, the investigators studied physicochemical and pharmacological properties of chitosan-coated PLGA nanoparticles and non-coated PLGA nanoparticles unloaded or loaded with exendin-4 (Ex4) or Ex4-C_{16}. Particle sizes and zeta potentials were determined my means of a Zetasizer and a He-Ne laser, whereas field emission scanning electron microscopy (FE-SEM) and transmission electron microscopy (TEM) served for analysis of surface morphologies. The release profiles of Ex4 or Ex4-C_{16} from

loaded nanoparticles were determined by means of an FPLC method. Both the adhesion characteristics and the toxicity of the nanoparticles were analyzed *in vitro* in the model of A549 cells. The nanoparticles were aerosolized using a microsprayer analyzer and the consecutive imaging was performed with a digital camera. Pulmonary deposition of coumarin 6-labeled chitosan PLGA nanoparticles was determined in ICR mice up to 72 h after intratracheal insufflation with a microsprayer. The hypoglycemic effect of Ex4- or Ex4-C_{16}-loaded chitosan PLGA nanoparticles was determined in db/db mice for up to 96 h after administration. The results demonstrated a slightly higher size and a significantly higher zeta potential of chitosan-coated *vs.* non-coated PLGA nanoparticles (695.7 ± 62.7 nm *vs.* 593.7 ± 33.3 nm and 28.5 ± 0.4 mV *vs.* -17.9 ± 0.5 mV, respectively). As shown by FE-SEM and TEM both types of particles were compact and spherical with particle sizes as described before. In the release experiments, incorporated Ex4 was rapidly released without a difference between chitosan-coated and non-coated PLGA nanoparticles (>80 % after 1 day and >90 % after 2 days). In contrast, the release of incorporated Ex4-C_{16} was much slower than that of Ex4 and the difference was most obvious for chitosan-coated PLGA nanoparticles (chitosan-coated *vs.* non-coated PLGA nanoparticles; 10 % *vs.* 30 % after 1 day and 60 % *vs.* 90 % after 2 days, respectively). Imaging of the aerosolization process demonstrated a good mobility of chitosan-coated nanoparticles which was obviously not affected by loading of Ex4 or Ex4-C_{16}. Incubation of A549 cells with coumarin 6 labeled chitosan-coated or non-coated PLGA nanoparticles demonstrated a largely higher adherence of the chitosan-coated PLGA nanoparticles. The investigation of cytotoxicity in the same cell line demonstrated no cytotoxic effect after a 12 h incubation period at low concentration (0.003 mg/ml) irrespective of chitosan-coating of the PLGA nanoparticles. However, a mild cytotoxic effect was observed for high concentrations (>0.03 mg/ml) also irrespective of chitosan-coating. After intratracheal insufflation of coumarin 6-loaded chitosan-coated or non-coated PLGA nanoparticles in ICR mice, the administered nanoparticles were rapidly deposited. However, chitosan-coated nanoparticles were gradually eliminated over about 72 h, whereas non-coated nanoparticles were rapidly cleared within 8 h. The investigation of the hypoglycemic effects after administration of Ex4- or Ex-C_{16}-loaded chitosan-coated PLGA nanoparticles in comparison with blank PLGA nanoparticles was performed in db/db mice. The nadir of the blood glucose concentration was observed 4 h and 24 h after dosing Ex4- or Ex-C_{16}-loaded chitosan coated PLGA nanoparticles, respectively. However, administration of Ex-C_{16}-loaded particles was followed by a longer hypoglycemic effect (baseline value about 400 mg/dl; Ex4- *vs.* Ex-C_{16}-loaded chitosan-coated PLGA nanoparticles; >400 mg/dl *vs.* 206.5 ± 74.9 mg/dl after 48 h, respectively). In addition, the induced hypoglycemia was 3.1-fold greater after administration of Ex-C_{16}-loaded chitosan-coated PLGA nanoparticles ($17{,}653.6 \pm 2{,}947.7$ mg × h/dl *vs.* $5{,}669.3 \pm 587.2$ mg × h/dl). In summary, the study demonstrated an improved mucoadhesive effect due to chitosan-coating of the PLGA particles, the sustained release effects of PLGA and palmitic acid, and the longevity of released Ex-C_{16} in the circulation caused by the binding to albumin (Table 1).

A study of a different design investigated the effect of the GLP-1 analog ROSE-010 in Sprague-Dawley rats (Hellström et al. 2012). ROSE-010 (chemical name Val^{10}-GLP) is a synthetic GLP-1 analog which has a high affinity to the GLP-1 receptor. Compared with native GLP-1(1–37), valine is substituted for alanine at position 8, protecting the molecule against N-terminal proteolytic cleavage by DPP-4. In a first experiment, the effects of intravenous GLP-1, ROSE-010, and exendin(9–39)amide (a competitive GLP-1 receptor antagonist) on small bowel migrating myoelectric complex (MMC) and myoelectric spiking activity were investigated in rats. In four groups doses of 1.0, 10.0, and 100 μg/kg ROSE-010 or GLP-1 were given alone or in combination with exendin

(9–39)amide (at 300 μg/kg infusion). GLP-1 and ROSE-010 had a dose dependent effect on the MMC cycle length in all the doses. Doses of 100 μg/kg GLP-1 and ROSE-010 abolished all myoelectric spiking activity for 49.1 ± 4.2 or 73.3 ± 7.7 min, respectively. The corresponding MMC cycle length increased to 131 ± 11.4 min and 149.3 ± 15.5 min, respectively (baseline MMC cycle length: 17.5 ± 0.8 min). In contrast, concurrent infusion of exendin(9–39)amide reversed these effects of GLP-1 and ROSE-010. In a second experiment, ROSE-010 was administered by intravenous injection (100 μg/kg), subcutaneous injection (100 μg/kg), or pulmonary insufflation of 100 and 200 μg/kg Technosphere® powder by means of an air pump and an insufflation chamber, whereas administration of saline or air served as control. Both intravenous and subcutaneous injections were followed by a significant extension of the MMC cycle length, when compared with control (124.1 ± 27.3 min *vs.* 18.3 ± 2.5 min and 148.1 ± 49.4 min *vs.* 14.8 ± 2.0 min, respectively). Pulmonary administration of 100 and 200 μg/kg Rose-010 Technosphere® resulted in a similar response without a dose-dependent difference (102.6 ± 18.3 min *vs.* 19.4 ± 2.9 min and 105.9 ± 9.5 min *vs.* 18.7 ± 7.3 min). In summary, the investigators demonstrated that both GLP-1 and ROSE-010 had a similar effect on gut motility, and pulmonary administration of ROSE-10 had an effect comparable to intravenous or subcutaneous administration (Table 1).

4 Human Studies Investigating the Inhalation of GLP-1

There are few studies on the inhalation of GLP-1 in humans. GLP-1 was administered by means of Technosphere® particles, the drug delivery platform approved for insulin delivery, which may also serve for delivery of other biomolecules (Mannkind Pharmaceuticals 2014; Cassidy et al. 2011; Marino et al. 2010; Potocka et al. 2010). Microparticles of FDKP (bis-3,6 (4-fumarylaminobutyl)-2,5-diketopiperazine and polysorbate 80) served as the primary component of this system. Highly soluble in water at neutral and basic pH values, FDKP undergoes intermolecular self-assembly under acidic pH (<5.2), crystallizing to highly porous microparticles (internal porosity about 70 %) with a large surface area. Proteins or other compounds, with a median aerodynamic diameter of 2–2.5 μm (10 % of particles <1 μm and 90 % <5 μm), which is in the range for pulmonary deposition, can be absorbed on the surface (Cassidy et al. 2011; Scheuch and Siekmeier 2007). The pharmacokinetics of FDKP was studied by Potocka et al. (2010) who investigated healthy individuals and different patient groups. A first study was designed as an open-label, nonrandomized, two-period, fixed-sequence crossover absorption, distribution, metabolism and excretion (ADME) study. Six healthy non-smokers received single intravenous (10 mg) and oral doses (20 mg) of ^{14}[C]FDKP solution and serial sampling of blood, urine, feces and expired air were performed. Two more single-dose, open-label, parallel-design studies were performed, where diabetic patients with normal kidney function and with diabetic nephropathy (12 and 24 patients, respectively), and healthy individuals, and patients with chronic liver disease (12 individuals and 21 patients, respectively) inhaled 20 mg FDKP. In the ADME study, <90 % of total radioactivity was eliminated within 8 h after intravenous administration, of which 97.2 % were recovered in urine and 1.65 % in feces without evidence of metabolism. After oral dosing, >90 % of radioactivity was eliminated within the first 48 h, with 2.45 % of total radioactivity recovered in urine and 95.10 % in feces, and only 0.03 % of the dose in the expired air. Comparison of pharmacokinetics between diabetic patients with and without nephropathy revealed higher values of maximum drug concentration ($C_{max.}$; geometric mean (% coefficient of variation); 159.9 ng/ml (59 %) *vs.* 147.0 ng/ml (44 %)) and area under the curve between 0 and 480 min in patients with nephropathy ($AUC_{0–480}$; 36.869 ng/ml × min (47 %) *vs.* 30.474 ng/ml × min (32 %)). However, the differences were not clinically significant. There were also no significant differences

of these parameters between patients with chronic liver disease and healthy individuals ($C_{max.}$: 160.2 ng/ml (36 %) *vs*. 143.4 ng/ml (49 %); $AUC_{0–480}$; 31.477 ng/ml × min (29 %) *vs*. 26.710 ng/ml × min (35 %)). In summary, the results show no oral availability and no metabolism of FDKP, demonstrating the safety of Technosphere® even in patients with diabetic nephropathy and chronic liver disease.

Marino et al. (2010) performed a proof-of-concept study investigating the pharmacokinetics of inhaled GLP-1(7–36)amide (MKC253) and the pharmacokinetic-pharmacodynamic relationship between inhaled GLP-1 and insulin in two trials; one in healthy individuals and the other in type 2 diabetic patients. The first study included 26 healthy individuals and was a phase I, single-dose trial incorporating an open-label ascending-dose structure (five doses: 0.05, 0.45, 0.75, 1.05, and 1.5 mg GLP-1) for the determination of safety and tolerability. Blood parameters under study were GLP-1, FDKP, glucagon, glucose, insulin, and C-peptide before and up to 120 min after dosing. Dosing was performed with subjects in fasting condition, and after 125 min and 240 min the individuals were given a snack and a full meal, respectively. In the second study, 20 type 2 diabetic patients (average values; age: 60 years, duration of diabetes: 7.2 years, body mass index (BMI): 27.2, HbA_{1c}: 7.11 %, baseline glucose: 8.76 mmol/l, and serum creatinine: 94.1 μmol/l) were included. The study was a phase I, single dose, placebo- and active comparator-controlled, and randomized treatment design. In the treatment period, patients received five different treatments (I: 1.5 mg GLP-1 as MKC253 inhalation powder or Technosphere® inhalation powder with continued fasting after dosing; II: 1.5 mg GLP-1 as MKC253 inhalation powder immediately before and 30 min after the meal preceded by s.c. saline injection before the meal; III: 1.5 mg GLP-1 as MKC253 inhalation powder immediately before the meal and Technosphere® inhalation powder 30 min after the meal preceded by s.c. saline injection 15 min before the meal; IV: Technosphere® inhalation powder immediately before and 30 min after the meal preceded by s.c. saline injection 15 min before the meal (placebo comparison), V: Technosphere® inhalation powder immediately before and 30 min after the meal preceded by s.c. 10 μg exenatide injection 15 min before the meal (active comparison)) on days 1, 3, 5, 7, and 9. Blood parameters, as in the first study, were determined both prior to administration and up to 240 min after administration of MKC253. Gastric emptying was determined after treatments 2–5 by means of administration of a Na-^{13}C-octanoate labeled muffin and expiratory breath analysis for $^{13}CO_2$. GLP-1, delivered by MKC253, was rapidly absorbed with peak plasma concentrations within 5 min (K_a values of 2.62 min^{-1} and 1.26 min^{-1} in healthy individuals and diabetic patients, respectively) returning to baseline concentrations within 30 min in both study groups. The observed changes of GLP-1 concentration fitted with different pharmacological models in healthy individuals (two-compartment model with first-order absorption) and type 2 diabetic patients (one-compartment model with first-order absorption and first-order elimination). However, the estimated bioavailability for MKC253 was below 2 %, likely secondary to the inhaled fraction of the compound entering the lung and the pulmonary DPP-4 activity. In both study groups, the increase of GLP-1 plasma concentration in the fasting state was followed by a rapid increase of plasma concentrations of insulin and C-peptide which peaked within 10–15 min. Peak plasma concentrations of insulin in diabetic patients were similar in fasting and non-fasting patients demonstrating that sufficient GLP-1 concentrations can stimulate insulin secretion in the fasting state. In fasting individuals (healthy volunteers and diabetic patients) plasma insulin concentrations returned to baseline within 30–40 min; however, in diabetic patients who had a meal, meal-stimulated insulin concentrations were observed for several hours. The relationship between plasma concentrations of GLP-1 and insulin release was investigated by a maximum effect ($E_{max.}$) model demonstrating a relation in both study groups. However, in diabetic patients higher values of $E_{max.}$ were found

in patients with higher baseline glucose concentrations. In the group of healthy individuals, inhalation of GLP-1 was followed by a minimally suppressed secretion of glucagon. In the patient group treated with GLP-1, along with the meal challenge (^{13}C-labeled muffin), one inhalation of GLP-1 reduced early glucose excursion, whereas two inhalations within 30 min resulted in a larger reduction. However, no significant effect of GLP-1 inhalation on gastric emptying was observed, which stands in contrast to a prior study where GLP-1 was administered intravenously and the observed concentrations were similar (Little et al. 2006). The most frequent adverse reaction in both study groups was cough limited to the time period around dosing. In the patient group, adverse events were nausea after inhalation of MKC253 in one patient and nausea in three patients (of which one with vomiting) treated with exenatide. However, no significant hypoglycemia was observed in both study groups. In summary, the authors demonstrated the feasibility of GLP-1 inhalation by means of the Technosphere® platform resulting in a very physiological biological response and a low rate of adverse reactions (Marino et al. 2010) (Table 1).

Our review compiles a number of studies investigating inhalative administration of GLP-1 and GLP-1 analogs in animals and one feasibility study in humans using very different techniques for modification of the pharmacological properties and modes for drug administration. However, further studies are needed to demonstrate long-time safety of this therapy, as not only the modification of the pharmacological compound itself but even carriers and excipients may have harmful effects (Hussain et al. 2004; Kim et al. 2011a; Siekmeier and Scheuch 2008b). Currently, the best feasibility has been demonstrated for inhalation of GLP-1 by means of the Technosphere® platform which has been subject of intensive studies for inhalative administration of insulin and is now approved by the FDA (Mannkind Pharmaceuticals 2014; Siekmeier and Scheuch 2008a). Inhalation of GLP-1 or its analogs may serve as a potential treatment in the large number of patients with type 2 diabetes mellitus without risk for hypoglycemia and an alternative for the required subcutaneous administration of these compounds. However, such therapy may also serve for treatment of patients with irritable bowel syndrome (RBS). Major advantages of inhalation therapy are the non-invasive character of drug administration, the lower presystemic metabolism of the inhaled drug, and the mimicking of the physiological profile of drug action resulting in the avoidance of adverse reactions and an improvement of therapeutic efficiency and convenience for patients.

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

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The Influence of Particulate Matter on Respiratory Morbidity and Mortality in Children and Infants

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Abstract

Air pollution is the most important environmental health risk leading to premature mortality, respiratory and other health problems. The aim of this study was to quantify its impact on infants and children in Warsaw (Poland), following the principles of Health Impact Assessment method. Particulate matter ($PM_{2.5}$ and PM_{10}) was considered as the indicator of air pollution. Exposure-response functions between air pollution and health impacts were employed based on the literature. According to the calculations, around 5,201 asthma symptoms and 234 hospital respiratory admissions were caused annually due to air pollution. Hospitalizations due to cardiovascular problems related to air pollution amounted to 13. The mortality among infants and children is relatively low and occurs mostly in the postneonatal period. Nonetheless, approx. 5 mortality cases were assessed to be air pollution-attributable. The study demonstrates a significant impact of air pollution on infants and children, which is manifested primarily as a range of respiratory problems.

Keywords

Air pollution • Children • Health Impact Assessment • Infants • Particulate matter • Respiratory diseases

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1 Introduction

A negative effect of air pollution on human health has been widely recognized. According to World Health Organization (2009), urban outdoor air pollution was worldwide the 14th of the 19 leading risk factors for mortality in 2004. A recent comparative risk assessment of burden of disease in 21 regions in the world (Lim et al. 2012) has demonstrated approx. 3.2 million deaths and more than 76 million disability-adjusted life-years related to air pollution in 2010. Scientific evidence of the increasing levels of exposure to air pollution (and particulate matter especially) and a growing risk of lung cancer led the International Agency for Research on Cancer (IARC) in 2013 to classify outdoor air pollution (and PM separately) as carcinogenic to humans (WHO 2013a). The WHO indicated that, globally, 3.7 million deaths are attributable to ambient air pollution, basing on the 2012 data. It is also estimated that the joint effect of household and ambient air pollution causes seven million of premature deaths yearly (WHO 2014).

Air pollution is the most important environmental health risk, which is supported by numerous epidemiological studies (Krzyzanowski et al. 2014; Beelen et al. 2014; Pope et al. 1995; Dockery et al. 1993). In particular, particulate matter (PM) air pollution is a major public health concern in urban areas (Brugha and Grigg 2014). PM consists of a mixture of solid and liquid particles suspended in the air that vary in number, size, shape, surface, area, chemical composition, solubility, and origin. Particle size is usually defined relative to a 50 % cut-off point at a specific aerodynamic diameter (such as 2.5 μm or 10 μm). In most locations in Europe, $PM_{2.5}$ constitutes 50–70 % of PM_{10} (WHO 2013b). The health effects of both short- and long-term exposure to PM_{10} and $PM_{2.5}$ are well documented, with no evidence of a safe level of exposure or a threshold below which no adverse health effects occur (Pope and Dockery 2006).

There is now substantial evidence that ambient air pollution is associated with increased mortality and morbidity in children (e.g., Beatty and Shimshack 2014; Lacasaña et al. 2005; Ward and Ayres 2004). The special vulnerability of children to exposure to air pollution is related to several differences between children and adults (WHO 2005), *inter alia*, the ongoing process of lung growth and development, incomplete metabolic systems, immature host defenses, and high rates of infection by respiratory pathogens. Moreover, children spend more time outdoors than adults, which increases their direct outdoor exposure (Schwartz 2004). Some authors also raise the issue of the implications of these air pollution-attributable health problems in youth, which may have long-lasting effects by impeding long-term human capital development (Beatty and Shimshack 2014).

The aim of this study was to quantify the annual impact of air pollution on infants and children in Warsaw (Poland), following the principles of Health Impact Assessment (HIA). Poland is a country which according to an Europe-wide study performed by the European Environment Agency (EEA 2013) of 2011 was one of the most polluted countries in the European Union. Moreover, recent research, which has been completed in Warsaw, indicated that traffic-related air pollutants significantly increase the risk of bronchi obstruction among people living in the proximity of main roads, compared to residents of rural areas (Badyda et al. 2013, 2014).

2 Methods

2.1 General Approach

The method used in this study follows the general guidelines on the assessment and use of epidemiological evidence for environmental health risk assessment recommended by WHO (2000). It consists of several steps and is based on the Health Impact Assessment (HIA) approach. Firstly, the environmental exposure and its distribution in the target population need to be specified. In case of a mixed exposure, which is typical for air pollution, the most reasonable indicator (or a group of indicators) has to be carefully selected and discussed. Secondly, the appropriate health outcomes (i.e., endpoints, such as chronic respiratory diseases incidence and prevalence, respiratory

hospital admissions, etc.) are defined on the basis of estimated exposure and the availability of the necessary data. The next step involves adopting health risk estimates, i.e., exposure-response functions (ERFs), from epidemiological studies. These functions describe quantitatively the change of a specified health effect due to the change of exposure to a given amount of the specified agent. The information on ERFs can be obtained from pooled analyses or meta-analyses. After that, population baseline frequency measures for the selected health outcomes are derived in order to quantify the prevalence or incidence of the outcomes under consideration. Finally, the number of attributable cases is calculated, assuming that exposure gives rise to the health outcome – the relation explained by the distribution of the exposure in the target population, the adopted ERFs and the estimated baseline frequency of the health outcome in the target population (Krzyzanowski et al. 2002; WHO 2000). The impact assessed by means of HIA can be further economically evaluated (e.g., Jakubiak-Lasocka et al. 2014; Künzli et al. 1999) in order to help the decision-makers to judge the potential negative health effects of a policy, program, or project concerning specific population.

As implied above, the procedure for quantification of the expected health effects related to air pollution requires many assumptions and methodological decisions to be taken and large number of data to be collected. Therefore, to take into account an inherent uncertainty in the calculations, an 'at least' approach was applied at each step of this study. These methodological assumptions led to the results expected to be 'at least' attributable to air pollution (Künzli et al. 1999, 2000). In this way the impact of air pollution obtained in this study is expected to be underestimated rather than overestimated.

2.2 Population Exposure

Air pollution contaminates the atmosphere by a mixture of substances present at concentrations above their normal ambient levels, which produces undesirable effects on human, animals, vegetation, or materials (Seinfeld and Pandis 2006). Pollutants of major public health concern include particulate matter, carbon monoxide, ozone, sulfur dioxide, nitrogen oxides, and volatile organic compounds (Kampa and Castanas 2007; Künzli et al. 1999). The pollutants are often highly correlated. It is, therefore, impossible to strictly allocate observed effects to single pollutants. A pollutant-by-pollutant assessment would result in a significant overestimation of the health impact. The usual approach in the air pollution epidemiology consists of a selection of only one pollutant to be an indicator of the complex mixture (Künzli et al. 1999, 2000). In the present study, $PM_{2.5}$ and PM_{10} were considered as a source of attributable cases. The reason for that was, above all, the most convincing evidence for adverse health effects existing for PM (Brugha and Grigg 2014), which enabled the widest possible approach to the subject. Another point was that PM has been commonly used in other studies to assess the impact of air pollution on human health.

The exposure of infants and children in the city of Warsaw to $PM_{2.5}$ and PM_{10} was evaluated on the assumption that the whole population within the city borders has been exposed to the same level of air quality, indicated by the pollutant measurement data from outdoor monitoring sites. This is due to the lack of data that would allow to assign a particular part of the population to a specific monitoring site. The health effects of air pollution, depending on the time between the exposure and effect, can be divided into two broad categories: long-term and short-term (Pope and Dockery 2006). In both cases, the distribution of infants and children's exposure to the average annual concentration of $PM_{2.5}$ and PM_{10} was employed, rather than the exposure distribution for each and every day. To be in line with the methods used in previous studies (Künzli et al. 1999, 2000), it was assumed that the average level of particular pollutant at any day, corresponds to its annual mean level. Consequently, the sum of all daily effects across one year corresponds to the annual impact.

The annual average level of ambient $PM_{2.5}$ and PM_{10} for years 2011–2013 (Fig. 1) was derived from results of direct measurements

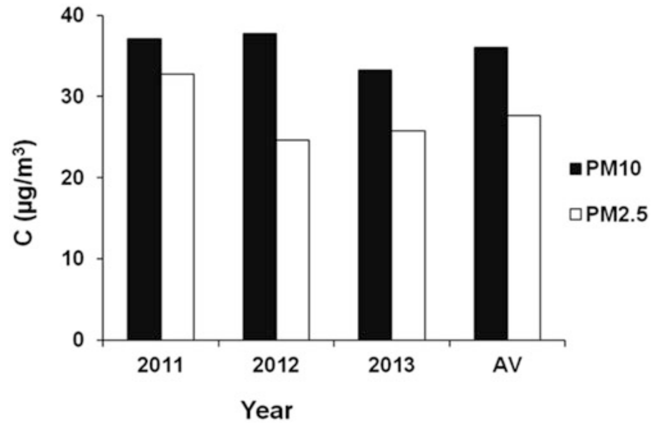

Fig. 1 Annual average concentration of $PM_{2.5}$ and PM_{10} for the years 2011–2013 and their average (AV)

carried out by the Regional Inspectorate of Environmental Protection in Warsaw (WIOS 2014). The monitoring system of air quality in Warsaw consists of 11 automatic and manual monitoring sites (as of 2014), eight of which are capable of measurement of PM_{10} level and four of $PM_{2.5}$. Yet it must be emphasized that the measurements from all the monitoring sites were not available, due to some internal problems leading to cancellation of the results and consequently not publishing them by WIOS. Thus, data on the concentration of PM_{10} ($PM_{2.5}$) came from six (four) monitoring sites in 2011, four (three) in 2012, and five (three) in 2013.

2.3 Health Effects and Risk Estimates

As already mentioned, there is large evidence in the literature over the negative influence of particles on various health outcomes among infants and children. Therefore, it seems substantial to integrate as much of the available information as possible by quantitative analyses of the results of individual studies. In the present study, the endpoints and the estimates of their effects in the form of relative risk (RR) for a given change in exposure were derived from ENHIS-1 (Environment and Health Information System – phase 1) – part of the APHEIS project (Air Pollution and Health: A European Information System) – which pays special attention to children, including the effects of PM_{10} on, *inter alia*, postneonatal mortality, hospital respiratory admissions, cough, and lower respiratory symptoms. The authors of this project were using the following criteria (in a graded order) in the selection process of RR:

- summary estimates from meta-analysis
- original studies involving large populations
- interrelated outcomes with high overall evidence of a causal contribution of air pollution.

It is worth noticing that this project was conducted in 31 European cities and one of its participant was Polish city of Cracow (APHEIS (ENHIS) 2005).

The estimates selected from the APHEIS project were completed with the data recently recommended by WHO (2013c), as part of HRAPIE (Health Risks of Air Pollution in Europe) project: recommendations for concentration—response functions for cost–benefit analysis of particulate matter, ozone, and nitrogen dioxide. After the elimination of overlapping health effects, the scope of this study was widened to the following effects of PM_{10} and $PM_{2,5}$: all-cause mortality, hospital cardiovascular admissions, prevalence of bronchitis and incidence of asthma symptoms in asthmatic children. To conclude: from both projects all endpoints measuring the effects of particles on health of infants and children were derived and included in the present study.

All the selected health effects, with the defined populations, effect estimates (RR for 10 μg/m^3 increase with 95 % CI (Confidence Interval)), and the original source of ERFs are presented in Table 1. The effect estimates for the endpoints: hospital respiratory admissions

Table 1 Health outcomes with relative risks and data sources

Health effect	Population age group	Pollutant	RR for 10 μg/m^3 increase (95 % CI)	Source of ERFs	Source of health data
Total postneonatal mortality	1 month-1 year	PM_{10}	1.048 (1.022; 1.075)	Lacasaña et al. (2005)	CWO (2013)
Postneonatal respiratory mortality			1.216 (1.102; 1.342)		CWO (2013)
Postneonatal SIDS mortality			1.120 (1.070; 1.170)	Woodruff et al. (1997)	CWO (2013)
Cough	5–17 years		1.041 (1.020; 1.0511)	Ward and Ayres (2004)	n/a
Lower respiratory symptoms			1.041 (1.020; 1.617)	Ward and Ayres (2004)	n/a
Hospital respiratory admissions	<15 years		1.010 (0.998; 1.021)	Anderson et al. (2004)	CWO (2013)
Prevalence of bronchitis in children	6–12 (or 6–18) years		1.080 (0.980; 1.190)	Hoek et al. (2012)	n/a
Incidence of asthma symptoms in asthmatic children[a]	5–19 years		1.028 (1.006; 1.051)	Weinmayr et al. (2010)	CWO (2013) and Komorowski (2012)
Mortality, all-cause	All ages	$PM_{2.5}$	1.012 (1.005; 1.020)	WHO (2013c)	CWO (2013)
Hospital cardiovascular admissions			1.009 (1.002; 1.017)	WHO (2013c)	CWO (2013)
Hospital respiratory admissions			1.019 (0.998; 1.040)	WHO (2013c)	CWO (2013)

[a]Total person-days with asthma attacks
SIDS sudden infant death syndrome, *RR* relative risk, *ERF* exposure-response function

and prevalence of bronchitis in children were not statistically significant. However, the point estimates and the majority of the area covered by the confidence intervals demonstrated a negative influence of particles. In addition, a probabilistic sensitivity analysis was conducted.

2.4 Health Data

Most of the data on the prevalence or incidence of the selected outcomes in the target population were derived from a report of the city of Warsaw Office *The health status of residents of Warsaw in 2009–2011* (CWO 2013). As the data were reported for 3 years, the average was incorporated into calculations, in order to obtain a more robust annual estimation, neglecting some eventual singular deviations. Unfortunately, not the whole health data were available and therefore, the health effects such as cough, lower respiratory symptoms, and the prevalence of bronchitis in children were not taken into account in the evaluation. That obviously underestimates the results obtained. It should also be emphasized that the population age-groups overlapped for the selected endpoints. For instance, assessing the attributable cases of hospital respiratory admissions among children under 15 years of age and hospital respiratory admissions among all children (all ages) would lead to an overestimation. Therefore, some age-group adjustments were performed (Table 1, column: population age-group *vs.* Table 2, column: population age-group in HIA).

The whole required health data were not presented in the report (CWO 2013) in the form that would allow their immediate use in the calculations. Therefore, for the endpoints connected with hospitalizations, the number of all hospitalized children (by relevant age-groups) was multiplied by the fraction of children hospitalized due to respiratory or cardiovascular reasons. Similarly, the data on postneonatal deaths were presented for infants aged 0–12 months, whereas the population age-group

Table 2 Annual impact of particulate matter (PM) on health of infants and children

Health outcome	Population age groups in HIA	Number of cases (population)	PM-attributable number of cases
Total postneonatal mortality including:	1 month-1 year	25	4
Postneonatal respiratory mortality		1	0
Postneonatal SIDS mortality		4	1
Mortality, all-cause	1–18 years	42	1
Incidence of asthma symptoms in asthmatic children[a]	5–18 years	56,733	5,201
Hospital respiratory admissions	<15 years	5,551	193
Hospital respiratory admissions	15–18 years	824	41
Hospital cardiovascular admissions	0–18 years	510	13

[a]Total person-days with asthma attacks

HIA Health Impact Assessment, *SIDS* sudden infant death syndrome

in the epidemiological studies was 1–12 month old. However, it was possible to derive a ratio of cases in first 1–12 months to 0–12 months (amounting to approx. 30 %) and adjust the data. Nonetheless, in case of sudden infant death syndrome (SIDS), the data were not adjusted, as the peak of its incidence occurs at 2–4 months of age (Kinney and Thach 2009). The use of the ratio would then definitely underestimate the result, so it was assumed that all four cases happened after the first month of life. The incidence of asthma symptoms in asthmatic children, as an endpoint, was calculated in two stages. Firstly, the number of asthmatic children in Warsaw was calculated as a product of all children in Warsaw and the percentage of diagnosed asthmatics (the average of age-groups: 6–7 and 13–14 years) in Warsaw (above 10 %) from a large Polish ECAP (Epidemiology of Allergic Diseases) study (Komorowski 2012). Secondly, the definition of the outcome regarding asthma symptoms (i.e. wheezing, shortness of breath, and asthma attacks) varied among the studies included in the meta-analysis (Weinmayr et al. 2010), from which the effect estimate was derived. In the present study the indicator for asthma symptoms were asthma attacks. However, due to the lack of such data for the Polish population, it was assumed that each asthmatic child, on average, suffers from three attacks a year. Such an assumption, based on the epidemiological data from other European countries, was adopted in a study by Künzli et al. (1999).

2.5 Number of Cases Caused by Air Pollution

The following formula was applied to obtain the number of cases caused by air pollution (n)[1]:

$$n = \frac{N}{1 + \frac{1}{(RR-1)\cdot\frac{E}{10}}}$$

where:

N – the total number of cases observed in the population of infants and children in Warsaw,

E – the exposure for infants and children in Warsaw (μg/m^3),

RR – relative risk selected from epidemiological studies.

3 Results

The mortality among infants and children was relatively low and occurred mostly in the postneonatal period (approx. 25 and 42 cases per year among infants aged 1–12 months and children

[1] For the derivation of this formula see: Kuschel et al. (2012).

Table 3 Probabilistic sensitivity analysis: particulate matter (PM) -attributable number of cases

Health outcome	Population age groups in HIA	PM-attributable number of cases		
		Median	5th percentile	95th percentile
Total postneonatal mortality	1 month-1 year	4	2	5
Postneonatal respiratory mortality		0	0	1
Postneonatal SIDS mortality		1	1	1
Morality all-cause	1–18 years	2	1	3
Incidence of asthma symptoms in asthmatic children[a]	5–18 years	5,173	1,786	8,203
Hospital respiratory admissions	<15 years	194	7	371
Hospital respiratory admissions	15–18 years	53	4	96
Hospital cardiovascular admissions	0–18 years	16	5	27

[a]Total person-days with asthma attacks
HIA Health Impact Assessment

aged 1–18 years). There were around 6,375 and 824 hospitalizations per year among children in Warsaw due to respiratory and cardiovascular problems, respectively. The incidence of asthma symptoms in children was high – assuming only three asthma attacks a year per asthmatic child – approx. 56,733 symptoms happened in a single year. The conducted HIA allowed to attribute some mortality/hospitalization cases to the particulate pollution. It was calculated that approx. 5 mortality cases were PM-attributable. Almost 5,201 asthma symptoms and 234 hospital respiratory admissions were caused by particulates. Hospitalizations due to cardiovascular problems related to the particulate pollution numbered 13. Table 2 presents the results – the total number of cases and the PM-attributable cases among all infants and children in Warsaw.

The results of the probabilistic sensitivity analysis: medians with percentile confidence intervals are depicted in Table 3.

4 Discussion

The aim of this study was to quantify the annual influence of air pollution on infants and children in the city of Warsaw in Poland. A summary of the estimates derived from the literature demonstrates a negative health influence, in particular on respiration. Overall, the health risk seems not be enhanced more than just a couple of percentage points, except for the risk related to postneonatal respiratory mortality which amounts to 21 %. However, the magnitude of the population exposed to this risk must be considered.

In case of infants and children, the absolute numbers connected with particulate-attributable deaths are not high, as children's mortality is generally low. However, the impact of PM on respiratory problems – asthmatic symptoms and hospitalizations – is considerable. It is worth emphasizing that the respiratory diseases are one of the most frequent reasons for hospital admissions among children. These diseases are also the most frequent cause, after postpartum complications, affecting the health status and requiring contact with health services.

The present study has limitations. The first problem concerns the transferability of the ERFs, derived from epidemiological studies, in which population of infants and children differed from the Polish one. The site-specific chemical composition of particulate matter also is different in various places. However, it seems that the use of meta-analysis of different studies for deriving the relative risks attenuates these problems. Finally, the assumption that the whole target population is exposed to the annual PM concentration may be easily criticized, since this idea fails to capture the relationship between the health of individuals in a population and the quality of air as measured at a fixed sampling point (e.g. Fisher et al. 2002). Nonetheless, the epidemiological studies have also been conducted

among children who had different outdoor activity and who were exposed to different air quality.

This study followed the ‘at least approach’ and therefore only not-overlapping endpoints assessing the impact of particulates (and no other pollutants) on health were considered, so that no double-counting is present. The main reason for choosing PM as an indicator of air pollution is the credibility and recognition of the epidemiological studies, which the ERFs are based on. Although there is growing evidence for the impact of other pollutants on children’s health, a pollutant-by-pollutant assessment would grossly overestimate the impact.

Moreover, it was not possible to include in the assessment all the health effects found in the literature due to the lack of relevant health data for the target population. Generally speaking, the morbidity data in Poland are not directly available from health statistics. Even the prevalence of asthma among children in Warsaw is difficult to assess. According to a large national study ECAP (Komorowski 2012) asthma is diagnosed in 10 % of children undergoing medical examination, whereas self-diagnosed asthma in that study amounted to approx. 6 %. However, according to a report on the health status of Warsaw residents (CWO 2013), only 37 out of 1,000 children have been diagnosed with asthma. That means that respiratory problems are generally not well recognized and it is difficult to make an assessment based on these data. Finally, the relationship between the pollution and health outcomes for non-infant children is still understudied and relatively poorly understood (Beatty and Shimshack 2014). Summing up, it seems that the presented influence can be in reality much underestimated.

5 Conclusions

The current knowledge is sufficient to state that air pollution adversely affects human health and in particular infants and children, causing unnecessary loss, pain, and generating costs borne by the whole society. Quantification of this impact has become an increasingly important component in policy discussion. The present study demonstrates a significant impact of air pollution on infants and children in Warsaw, which is manifested primarily as a range of respiratory problems. Yet the most effective means of protecting people from environmental health threats is exposure prevention. Therefore, more attention should be paid for the integrated environmental health policy, with a focus on children and infants as priority.

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Relationship Between History of Smoking, Metabolic and Inflammatory Markers, Parameters of Body Composition and Muscle Strength

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Abstract

The objective of this study was to investigate the relationship between smoking history expressed by pack-years, metabolic and inflammatory markers, parameters of body composition (BC) and muscle strength among heavy smokers. A detailed smoking history was obtained from 49 heavy smokers (age = 44 ± 12, pack-years = 31 ± 23). Blood samples were analyzed for levels of glucose, lipids, liver enzymes and C-reactive protein (CRP). Anthropometric measurements included waist circumference and assessment of BC by dual energy X-ray absorptiometry (DEXA) and bioelectrical impedance analysis (BIA). Muscle strength was assessed by handgrip dynamometry and predicted one-repetition maximum (p1RM) tests. Positive correlations were found between pack-years of smoking, fasting glucose, alkaline phosphatase and CRP levels. Pack-years were also positively correlated with waist circumference, body mass index (BMI), whole-body and trunk fat mass measured by both DEXA and BIA. A negative correlation was found between pack-years of smoking and muscle strength measured by p1RM for the leg press exercise. After adjustment for age, sex and BMI, a positive correlation remained between pack-years of smoking and CRP levels. In conclusion, after controlling for possible confounders, smoking history was found to be positively associated with CRP levels among heavy smokers.

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Keywords
Adipose tissue • Bioelectrical impedance analysis • Cigarette smoking • C-reactive protein • Dual energy X-ray absorptiometry • Pack-years • Skeletal muscle

1 Introduction

Despite the growing awareness regarding the health risk of smoking, approximately 20 % of the world population still smokes tobacco (Basu et al. 2011). Cigarette smoking is considered as one of the main causes of preventable illness and premature death worldwide (Gonçalves et al. 2011). Smoking is a well-documented risk factor for numerous pathologies including cardiovascular disease (CVD), chronic obstructive pulmonary disease (COPD) and type 2 diabetes mellitus (DM) (Fagard and Nilsson 2009; Swan and Lessov-Schlaggar 2007). In addition, smoking was identified as a risk factor for sarcopenia, an impaired state of health characterized by an age-related loss of skeletal muscle mass and strength, leading to physical disabilities, increased risk of falls, fractures and death (Cruz-Jentoft et al. 2010; Lee et al. 2007; Szulc et al. 2004; Castillo et al. 2003).

Some of the adverse consequences of cigarette smoking are represented by alterations in various metabolic and inflammatory markers. Smoking has been shown to accelerate the onset of type 2 DM by deteriorating glucose metabolism and aggravating insulin resistance (Fagard and Nilsson 2009). Also, smoking increases CVD risk by promoting an altered lipid profile of higher levels of total cholesterol, triglycerides (TG) and low-density lipoprotein (LDL) cholesterol as well as lower levels of high-density lipoprotein (HDL) cholesterol (Chelland Campbell et al. 2008; Craig et al. 1989). In addition, the pro-inflammatory impact of smoking, reflected by increased levels of cytokines and inflammatory markers in smokers, can partially explain the increased CVD risk caused by smoking (Rom et al. 2013; Dietrich et al. 2007). For instance, serum levels of C-reactive protein (CRP), an indicator of systemic inflammation and a predictor of increased coronary risk, were found to be elevated among smokers in comparison with non-smokers (Lao et al. 2009).

Previous studies suggest that smoking is associated with an altered body composition (BC) of low body weight and lean mass (LM) on the one hand, and increased abdominal adiposity on the other hand (Rom et al. 2014a). This altered body composition may also increase the risk for various pathologies common in smokers. Population studies have demonstrated that although smokers are leaner and have lower body mass index (BMI), smokers have higher waist-to-hip ratio when compared with non-smokers, indicating an adverse distribution of body fat toward central obesity (Clair et al. 2011; Canoy et al. 2005; Barrett-Connor and Khaw 1989). Abdominal fat accumulation seen among smokers can increase the risk of CVD and type 2 DM (Clair et al. 2011; Fagard and Nilsson 2009). In addition, older smokers were found to have lower LM when compared with never-smokers, suggesting that smoking may increase the risk for sarcopenia by enhancing muscle wasting and accelerating the decline in physical functioning (Van den Borst et al. 2011; Cruz-Jentoft et al. 2010; Lee et al. 2007; Szulc et al. 2004; Castillo et al. 2003).

As demonstrated in the above studies, some health risks of smoking can be mediated by alterations in metabolic and inflammatory markers, body composition (BC) and physical function. Therefore, investigating the relationship between smoking history and these parameters is of great importance. The current study aims to evaluate the association between history of smoking expressed by pack-years, metabolic and inflammatory markers, parameters of BC and muscle strength in a representative sample of adult heavy smokers.

2 Methods

2.1 Subjects

Approval for the study was obtained from the Helsinki Committee of Rambam Health Care Campus, Haifa, Israel. Subjects were recruited from the smoking cessation program of Clalit Health Services, Haifa and Western Galilee district, Israel. Eighty-one subjects were enrolled after signing informed consent. Inclusion criteria included heavy smoking (>15 cigarettes/day) and age range between 20 and 65 years. Exclusion criteria included CVD, COPD, DM, cancer, untreated thyroid disorder, use of corticosteroids, BMI >40 kg/m^2, and consumption of more than two alcoholic drinks per day. Medical history was provided by the family physician for each participant. Forty-nine subjects met the inclusion and exclusion criteria and were included in the study.

2.2 Smoking Status

The subjects were interviewed for their smoking history, including the number of cigarettes smoked per day at different life periods. To evaluate the combined effects of smoking during a lifetime, pack-years were calculated from the total number of years spent smoking multiplied by the number of cigarettes smoked daily at various life periods.

2.3 Biochemical Analysis

Blood samples were taken after an overnight fast of at least 8 h and analyzed for levels of glucose, lipids, and liver enzymes using automated diagnostic equipment (Beckman Coulter AU5800, Brea, CA). CRP levels were measured by the immunoturbidimetric assay for the in vitro quantitative determination of serum CRP (Roche Diagnostics, Indianapolis, IN). The cut-point of CRP levels which is usually used to identify inflammation is 1 mg/dl (Tracy et al. 1997). To exclude the effects of acute inflammation, CRP levels greater than 1 mg/dl, which were found in one subject, were not included in the analysis of the association between pack-years of smoking and CRP levels.

2.4 Body Composition Assessment

BC was assessed at the Department of Nuclear Medicine, Rambam Health Care Campus. Measurements were obtained in the morning after a fast of at least 1.5 h. Bio-electrical impedance analysis (BIA) of whole-body and segmental BC was performed with the BC-545 body composition monitor (Tanita Corporation, Tokyo, Japan) as previously described (Rom et al. 2014a). Also, whole-body and regional BC were estimated by dual-energy X-ray absorptiometry (DEXA) (Hologic, Bedford, MA), the reference method for BC assessment (Thibault et al. 2012), as previously described (Rom et al. 2014a, b). Height was measured by a standard wall-mounted measure (Seca 206, Birmingham, UK). Waist circumference was measured using a standard measuring tape at the superior iliac while standing in a relax posture.

2.5 Muscle Strength Assessment

Muscle strength was examined by a certified fitness instructor at the fitness center of Rambam Health Care Campus as previously described (Rom et al. 2014b). Handgrip strength was measured by the Jamar hydraulic dynamometer (PC 5030JI, Sammons Preston, Bolingbrook, IL). Tests were repeated three times for each arm and the average result was calculated. Subsequently, predicted one-repetition maximum (p1RM) tests for the chest press (CP) and leg press (LP) exercises were performed with HOIST H4400 resistance machines (HOIST Fitness Systems, Poway, CA). The tests began with a familiarization and warm-up sets. Then, resistances were increased progressively, separated by a 2-min rest, until the subjects could perform only 10 or fewer correct repetitions.

Resistances and repetitions were recorded and used to estimate one-repetition maximum using the Brzycki equation (Brzycki 1998).

2.6 Statistical Analysis

Data were reported as means ± SD. Normality was assessed by the Kolmogorov-Smirnov test. Variables that did not fit a normal distribution were logarithmically transformed to correct for skewness of the distribution. The associations between pack-years of smoking and other variables were calculated using Pearson's correlation and partial correlation after controlling for possible confounders including age, sex, and BMI. $p < 0.05$ was considered significant. Statistical analysis was performed by SPSS 18 software (SPSS Inc., Chicago, IL).

3 Results

3.1 Subjects Characteristics

Characteristics of the subjects, including age, pack-years of smoking, blood tests, parameters of BC and muscle strength, are depicted in Table 1.

3.2 Association Between Pack-Years of Smoking and Blood Parameters

The associations between pack-years of smoking and blood parameters examined in this study are presented in Table 2. Positive correlations were found between pack-years, fasting blood glucose, alkaline phosphatase (ALP) and CRP levels. After adjustment for age, sex and BMI, the above correlations remained only between pack-years of smoking and CRP levels. A scatter plot depicting the relationship between pack-years of smoking and CRP levels is presented in Fig. 1.

3.3 Association Between Pack-Years of Smoking, Parameters of Body Composition and Muscle Strength

The associations between pack-years of smoking, parameters of BC and muscle strength are presented in Table 3. Positive correlations were found between pack-years, waist circumference, BMI, whole-body and trunk fat mass (FM) measured by both DEXA and BIA. Negative correlation was found between pack-years of smoking and muscle strength measured by p1RM for the LP exercise. None of the above correlations remained after adjustment for age and sex.

4 Discussion

The aim of the present study was to investigate the relationship between history of smoking, metabolic and inflammatory markers, parameters of BC, and muscle strength among adult heavy smokers. Smoking history, expressed by pack-years of smoking, was found to be positively associated with fasting glucose, ALP, CRP, waist circumference, BMI, whole-body and trunk FM, and negatively associated with muscle strength measured by p1RM-LP. Interestingly, after adjustment for age, sex and BMI, the positive correlation remained solely between pack-years of smoking and CRP.

The main finding of the study was the positive correlation between smoking history and CRP levels that remained after controlling for possible confounders. CRP is one of most studied inflammatory markers. Numerous of studies have shown that CRP independently predicts cardiovascular events (Lao et al. 2009). Our findings are consistent with previous studies that found an association between smoking and CRP levels in large population studies. Most of these studies focused on the relationship between smoking status and CRP, while only a few evaluated this relationship in regard to a detailed smoking history. In a study of 2,920 British men, current smokers showed significantly higher levels of

Table 1 Subjects characteristics

Characteristics	Females (26) Mean ± SD	Females (26) Range	Males (23) Mean ± SD	Males (23) Range	Combined (49) Mean ± SD	Combined (49) Range
Age (year)	44.7 ± 12.5	26.0–64.0	42.7 ± 11.7	22.0–62.0	43.8 ± 12.0	22.0–64.0
Pack-years	31.5 ± 23.8	7.0–114.5	31.3 ± 23.5	3.6–90.0	31.4 ± 23.3	3.6–114.5
Glucose (mg/dl)[a]	88.2 ± 9.6	75.0–106.0	88.0 ± 8.5	74.0–112.0	88.1 ± 9.0	74.0–112.0
Cholesterol (mg/dl)[a]	186.0 ± 28.9	150.0–250.0	205.0 ± 48.0	138.0–300.0	194.6 ± 39.3	138.0–300.0
LDL-Cholesterol (mg/dl)[a]	112.1 ± 23.9	82.0–167.5	133.3 ± 41.9	70.3–204.2	121.3 ± 34.2	70.3–204.2
HDL-Cholesterol (mg/dl)[a]	52.6 ± 12.23	34.0–90.0	43.5 ± 9.4	31.0–65.0	48.5 ± 11.8	31.0–90.0
TG (mg/dl)[a]	106.3 ± 44.13	52.0–250.0	149.3 ± 67.0	64.0–315.0	125.7 ± 59.0	52.0–315.0
ALP (U/l)[a]	71.0 ± 17.7	46.0–107.0	79.8 ± 21.3	53.0–118.0	74.7 ± 19.5	46.0–118.0
AST (U/l)[a]	20.7 ± 7.5	14.0–29.0	25.1 ± 9.7	9.0–47.0	22.7 ± 7.4	9.0–47.0
ALT (U/l)[a]	18.7 ± 7.5	3.0–35.0	32.9 ± 18.6	12.0–69.0	25.1 ± 15.2	3.0–69.0
CRP (mg/dl)[b]	0.3 ± 0.6	0–2.8	0.2 ± 0.2	0–0.84	0.3 ± 0.5	0–2.8
Height (cm)	163.8 ± 7.5	150–176	176.5 ± 6.5	167–194	169.7 ± 9.5	150–194
Waist circumference (cm)	81.6 ± 13.4	62.5–106.0	88.2 ± 11.1	70.5–118.0	84.7 ± 12.7	62.5–118.0
BIA-Weight (kg)	67.1 ± 12.6	44.1–94.7	80.4 ± 13.6	59.7–122	73.3 ± 14.6	44.1–122
BIA-BMI (kg/m^2)	25.0 ± 4.4	16.8–32.5	25.7 ± 3.4	19.7–35.7	25.4 ± 4.0	16.8–35.7
BIA-FM (kg)	23.4 ± 9.8	5.8–44.3	17.3 ± 7.1	6.5–39.0	20.5 ± 9.0	5.8–44.3
BIA-FM (%)	33.4 ± 8.5	13.2–49.4	20.9 ± 5.5	10.9–32.0	27.5 ± 9.6	10.9–49.4
BIA-Trunk FM (%)	30.3 ± 10.4	5.0–49.8	22.6 ± 6.5	10.5–35.2	26.7 ± 9.5	5.0–49.8
BIA-LM (kg)	41.6 ± 4.0	35.3–51.3	59.9 ± 7.5	50.5–78.9	50.2 ± 10.9	35.3–78.9
DEXA-Total mass (kg)	66.1 ± 12.5	43.5–92.9	78.7 ± 13.1	59.1–118.1	72.0 ± 14.2	43.5–118.1
DEXA-BMI (kg/m^2)	24.7 ± 4.4	16.6–32.0	25.2 ± 3.3	19.5–34.5	24.9 ± 3.9	16.6–34.5
DEXA-FM (kg)	25.6 ± 8.7	9.5–43.0	21.1 ± 6.0	11.4–37.9	23.5 ± 7.8	9.5–43.0
DEXA-FM (%)	37.4 ± 6.9	21.7–50.1	26.4 ± 4.2	18.9–34.7	32.3 ± 8.0	18.9–50.1
DEXA-Trunk FM (%)	35.01 ± 8.6	17.9–50.5	27.2 ± 5.0	16.3–34.4	31.4 ± 8.1	16.3–50.5
DEXA-LM (kg)	38.5 ± 4.9	31.1–47.7	55.1 ± 7.9	45.3–77.2	46.3 ± 10.5	31.1–77.2
Handgrip strength (kg)	21.7 ± 6.1	11.3–33	44.6 ± 7.9	27.0–63.0	32.5 ± 13.4	11.3–63
p1RM-CP (kg)	25.3 ± 9.6	7.7–45.6	61.9 ± 12.7	39.7–85.3	42.5 ± 21.5	7.7–85.3
p1RM-LP (kg)	78.4 ± 19.5	39.4–115.1	124.2 ± 29.8	69.3–164.4	99.9 ± 33.7	39.4–164.4

LDL low-density lipoprotein, *HDL* high-density lipoprotein, *TG* triglycerides, *ALP* alkaline phosphatase, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *CRP* C-reactive protein, *BIA* bioelectrical impedance analysis, *BMI* body mass index, *FM* fat mass, *LM* lean mass, *DEXA* dual energy X-ray absorptiometry, *p1RM* predicted 1 repetition maximum, *CP* chest press, *LP* leg press

[a] Data is missing from 9 subjects (4 female, 5 male)

[b] Data is missing from 14 subjects (6 female, 8 male)

CRP when compared with never smokers (Wannamethee et al. 2005). Also, in a cross-sectional analysis of 2,999 Chinese men, CRP levels were found to increase linearly across never, former and current smokers (Lao et al. 2009). Finally, in a cross-sectional study of representative US survey data that included 3,505 men and 3,896 women, smoking was found to be associated with dose-dependent and time-dependent increases in CRP concentrations (Dietrich et al. 2007).

Table 2 Correlations between pack-years of smoking and blood parameters

Parameter	r	p	r[c]	p
Glucose[a]	**0.311**	**0.049**	0.033	0.845
Cholesterol[a]	0.049	0.762	−0.091	0.593
LDL-Cholesterol[a]	0.059	0.722	−0.054	0.753
HDL-Cholesterol[a]	−0.154	0.344	−0.084	0.620
TG[a]	0.148	0.361	−0.093	0.586
ALP[a]	**0.526**	**0.001**	0.066	0.707
AST[a]	−0.124	0.448	−0.098	0.565
Log (ALT)[a]	−0.216	0.181	−0.156	0.358
Log (CRP)[b]	**0.480**	**0.007**	**0.456**	**0.017**

LDL low-density lipoprotein, *HDL* how-density lipoprotein, *TG* triglycerides, *ALP* alkaline phosphatase, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *CRP* C-reactive protein, *r* correlation coefficient
$p < 0.05$ was considered significant (**bold**)
[a]n = 40
[b]n = 35
[c]After adjustment for age, sex, and BMI

Previous studies reported that smoking is associated with low body weight, on the one hand, and increased central obesity, on the other hand (Rom et al. 2014a; Clair et al. 2011; Canoy et al. 2005; Barrett-Connor and Khaw 1989). Thus, it was of interest to study the relationship between smoking history and parameters of body weight and fat distribution in our study sample. Also, lower levels of LM and muscle function were previously found in older smokers when compared with never-smokers (Van den Borst et al. 2011; Cruz-Jentoft et al. 2010; Lee et al. 2007; Szulc et al. 2004; Castillo et al. 2003). The measurement of LM and muscle strength in our study sample that included heavy smokers aged 20–65, allowed us to study the effects of smoking history on these parameters in a non-elderly population. Interestingly, pack-years of smoking were found to be positively associated with waist circumference, BMI, whole-body and trunk FM measured by both DEXA and BIA. We also found that pack-years of smoking were negatively correlated with

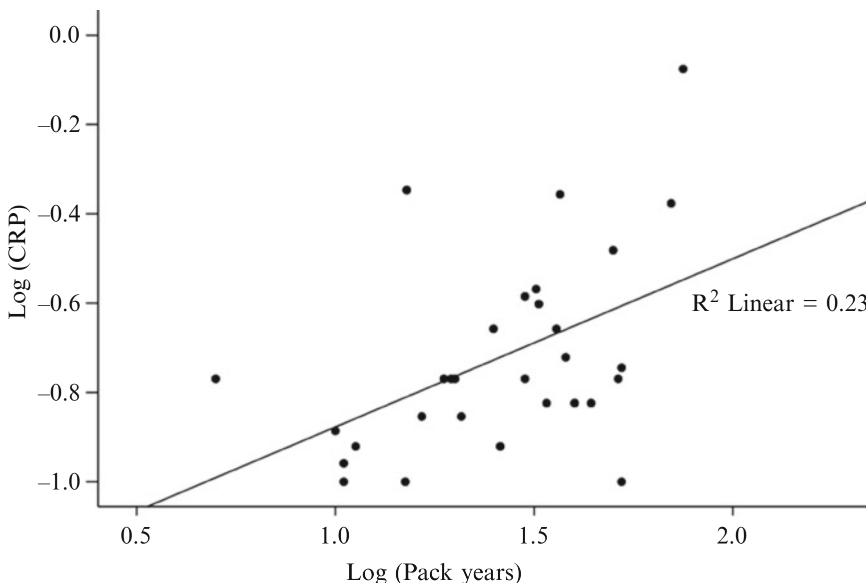

Fig. 1 Correlations between pack-years of smoking and CRP (C-reactive protein) levels

Table 3 Correlations between pack-years of smoking, parameters of body composition and muscle strength

Parameter	r	p	r[a]	p
BIA-Weight (kg)	0.239	0.098	0.070	0.639
BIA-BMI (kg/m^2)	**0.367**	**0.010**	0.022	0.884
BIA-FM (kg)	**0.427**	**0.002**	0.064	0.671
BIA-FM (%)	**0.397**	**0.005**	0.031	0.835
BIA-Trunk FM (%)	**0.454**	**0.007**	0.029	0.848
BIA-LM (kg)	0.001	0.993	0.064	0.671
DEXA-Total mass (kg)	0.243	0.092	0.069	0.644
DEXA-BMI (kg/m^2)	**0.372**	**0.008**	0.022	0.885
DEXA-FM (kg)	**0.385**	**0.006**	0.057	0.702
DEXA-FM (%)	**0.300**	**0.036**	−0.030	0.843
DEXA-Trunk FM (%)	**0.349**	**0.014**	−0.081	0.586
DEXA-LM (kg)	0.044	0.767	0.070	0.642
Waist circumference (cm)	**0.447**	**0.001**	0.054	0.717
Handgrip strength (kg)	−0.116	0.426	0.118	0.431
Log (p1RM-CP) (kg)	−0.188	0.197	−0.029	0.845
Log (p1RM-LP) (kg)	**−0.285**	**0.047**	−0.094	0.530

BC body composition, *BIA* segmental bioelectrical impedance analysis, *BMI* body mass index, *FM* fat mass, *LM* lean mass, *DEXA* dual energy X-ray absorptiometry, *p1RM* predicted 1 repetition maximum, *CP* chest press, *LP* leg press
$p < 0.05$ were considered significant (**bold**)
[a]After adjustment for age and sex

muscle strength measured by p1RM-LP, while no association was observed between smoking history and LM. However, after controlling for possible confounders, no correlations between pack-years of smoking, parameters of BC and muscle strength were found. In addition, it should be noted that although smoking is known to promote an adverse lipid profile of higher levels of total cholesterol, TG and LDL cholesterol, with lower levels of HDL cholesterol, (Chelland Campbell et al. 2008; Craig et al. 1989), we have not found any association between smoking history and lipid profile. The above findings may be explained by the relative small size and heterogeneity of our sample population in terms of age and gender. Therefore, future studies are warranted to investigate the association between history of smoking, body weight and fat distribution in larger and more homogenous study populations.

The main limitation of the current study was a relative small sample size, which limits the extrapolation of the findings to the entire population of smokers. The strength of this study includes the measurement of whole-body and segmental BC using both BIA and DEXA, which is considered the reference method for BC assessment (Thibault et al. 2012).

To conclude, after controlling for possible confounders including age, sex and BMI, pack-years of smoking were found to be positively associated with CRP levels in a sample of adult heavy smokers.

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

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Time and Dose Effects of Cigarette Smoke and Acrolein on Protein Carbonyl Formation in HaCaT Keratinocytes

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Abstract

Cigarette smoke (CS) is an important environmental source of human exposure to a highly toxic and chemically active α,β-unsaturated aldehyde: acrolein. It is capable of causing protein carbonylation and dysfunction, especially in oral tissues of smokers, constantly exposed to CS toxic constituents. The foremost damage is considered to be cumulative, but even a short exposure can be potentially harmful. The objectives of the current study were to examine the short time and dose effects of direct CS and acrolein exposure on intracellular protein carbonylation in epithelial cells. HaCaT-keratinocytes were exposed to different doses of acrolein and whole phase CS using a unique smoking simulator apparatus that mimics the exposure in smokers. The rate of intracellular protein carbonyl modification was examined 10–60 min after the exposure by Western blot. In addition, the effect of pre-incubation with a thiol scavenger N-acetylcysteine (NAC) was also assessed. We found that intracellular protein carbonyls increased as fast as 10 min after CS exposure and their concentration doubled after 20 min, with a slight elevation afterwards. Also, carbonyl levels increased gradually as CS and acrolein doses were elevated. Addition of 1 mM NAC neutralized part of the damage. We conclude that CS and acrolein intracellular protein carbonylation is dose- and time- dependent. Even a short time exposure to CS and its aldehydic constituents can be potentially harmful.

Keywords

Acrolein • Cigarette smoke • Keratinocytes • Protein carbonylation • Unsaturated aldehydes

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1 Introduction

Cigarette smoking is an established risk factor for diseases such as cancer, atherosclerosis, myocardial infarction, as well as periodontal disease (Reibel 2003; Kuper et al. 2002). Cigarette smoke (CS) is a mixture of countless chemically reactive components, affecting different organs (Kuper et al. 2002), and their molecular mechanisms of damage are far from being understood. CS is an important environmental source of human exposure to a highly toxic and chemically active unsaturated aldehyde: acrolein. It is the simplest unsaturated aldehyde (chemical name: propenal) and its α,β-double bond is highly electrophilic; hence the compound is highly toxic. Humans are exposed to acrolein mainly through environmental pollution such as automobile exhaust, but it is also present in various foods and formed during food heating and preparation. There is also internal production of acrolein through lipid peroxidation and metabolism of the amino acids methionine and threonine. Smoking of tobacco products equals or exceeds the total human exposure to acrolein from all other sources (Stevens and Maier 2008). Endogenously, acrolein can react with nucleophilic sites of macromolecules such as DNA, phospholipids and proteins. Reaction with proteins generates protein-carbonyl derivatives mainly on active sites of proteins such as -SH (thiol) groups of cysteine (Kehrer and Biswal 2000), but also histidine, arginine and lysine residues, inducing protein structural alteration (Uchida et al. 1998). This structural alteration, called protein carbonylation, may lead to protein dysfunction and may be followed by the initiation of pathologic processes and disease onset, including cancerous transformation (Nystrom 2005; Dalle-Donne et al. 2003; Kehrer and Biswal 2000). Enzymes, nuclear factors and antioxidants are especially sensitive protein targets.

Protein carbonylation is a constantly occurring process. It can be caused by endogenous oxidative products such as reactive oxygen and nitrogen species (ROS and RNS) generated under normal and pathologic conditions, and by exogenous sources such as CS (Cai and Yan 2013). Generally, modified proteins are not repaired and must be removed by proteolytic degradation, while some accumulate within the cell. The level of modified proteins can be measured and it has been used as a biomarker for the assessment of oxidative damage in aging and disease (Wehr and Levine 2013; Stadtman 2006). On the other hand, there is recent evidence that such modifications can play a positive role in cellular function and signal transduction under stress conditions (Cai and Yan 2013).

The tissues of the oral cavity are the first to face CS and its toxic elements entering the body. Smokers are at greater risk of developing various oral diseases including mucosal and periodontal inflammations, infections and oral cancer (Reibel 2003). Oral cavity tissues of smokers are constantly exposed to CS derived toxins, including acrolein. In saliva, CS exposure caused an increase in salivary protein carbonyls (Nagler et al. 2000). At least part of the effect can be attributed to CS aldehydes, especially acrolein (Avezov et al. 2014a). Concurrently, a decrease in the activity of some salivary enzymes such as lactate dehydrogenase (LDH), aspartate aminotransferase (AST), acid phosphatase and amylase was detected (Zappacosta et al. 2002; Nagler et al. 2001). Even if the effect of acrolein on saliva is short-term, since it is continuously secreted and replaced, a water soluble acrolein can penetrate into oral cells and cause intracellular carbonylation as well (Avezov et al. 2014b; Colombo et al. 2012). The primary target of acrolein toxicity are tissues at the site of contact, as was shown by irritation to the respiratory and gastrointestinal tracts, eyes, and skin following inhalation, oral, and dermal exposure (Gomes et al. 2002). α,β-unsaturated aldehydes are present in healthy subjects' saliva and airway secretions in low-micromolar concentrations, and are elevated up to tenfold in heavy smokers (Gomes et al. 2002). Despite its importance in oral pathology, the effects of acrolein on the oral cavity tissues have not been extensively investigated. Moreover, many studies focus on the importance of prolonged smoking in disease onset. The objectives of the current study were to

examine the short time and dose effects of CS and acrolein exposure on the intracellular protein carbonylation in keratinocytes.

2 Material and Methods

2.1 Cell Culture and Cell Viability Assay

HaCaT keratinocyte cell line used in the experiments was acquired from the CLS Cell Lines Service (Eppelheim, Germany). HaCaT are in vitro spontaneously transformed keratinocytes from histologically normal human skin (Boukamp et al. 1988). These cells are widely used as a model for epithelial tissue studies, including oral epithelium investigations, due to their high proliferation rate. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine, 10 % fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37 °C humidified atmosphere containing 5 % CO_2. All the experiments were executed in 90 % confluency, in 100 mm Nunclon cell culturing dishes submerged in 10 ml of cell culturing medium. Viability was assessed by the trypan blue exclusion method.

2.2 Exposure of Cell Cultures to CS and Aldehydes

The study was performed using filtered 'Time' cigarettes (Dubek, Israel, containing 14 mg of tar and 0.9 mg of nicotine per cigarette) combined with a source of lowered pressure system as previously described (Rom et al. 2013). In short: an open 100 mm Petri dishes with cell cultures submerged in 10 ml of culture medium were placed in a sealed reservoir with a sidearm to which a cigarette was attached. A reproducible low pressure was created in the reservoir by a vacuum pump. After the attached cigarette was lit, the sidearm was opened for 10 s and the smoke from the lit cigarette was drawn into the reservoir. This was considered a single 'puff'. The cells were exposed to an increasing (1–3) number of puffs and incubated for 1 h. In another experiments, the cell cultures were exposed to a single puff of CS, and incubated for different time intervals (10, 20, 40 and 60 min). Samples subjected to air puffs instead of CS were used as controls. The amount of CS drawn into the reservoir was regulated by the pressure inside the reservoir using a vacuum pump. Thus, the dose of CS entering the reservoir equated the level of lowered pressure created inside the reservoir as previously described (Avezov et al. 2014b). Immediately after the experiments, the cells were lysed, centrifuged and preserved in an ultra-low temperature freezer (−80 °C) for carbonylation assay for up to 15 days.

In separate experiments, cell cultures were incubated with 1 μmol of purified acrolein (Sigma-Aldrich; St. Louis, MO) added to cell medium in order to simulate exposure to acrolein content reported to be present in a mainstream smoke of one 2R1 University of Kentucky reference cigarette (O'Neill et al. 1994). To simulate a cumulative effect, a tenfold acrolein content was applied (10 μmol). Incubation conditions were similar to the cultures exposed to CS.

2.3 Detection of Protein Carbonyl Modifications

Commercially available OxyBlot Protein Oxidation Detection Kit (Millipore, Jaffrey, NH) was used as an indicator of protein carbonyl modification. The assay is based upon 2,4-dinitrophenylhydrazine (DNPH) carbonyl derivation, following immunodetection with the Western blot assay with anti-dinitrophenyl (DNP) antibodies and followed by quantification by densitometry.

2.4 Addition of N-Acetylcysteine (NAC) to Cell Medium

A thiol scavenger NAC 1 mM (Sigma-Aldrich, St. Louis, MO), capable of traversing cell membranes was added to the culture medium where noted and incubated with the cells for 1 h

prior to the experiments. Then, the medium was replaced (by a NAC free medium), and the experiments were conducted, verifying that thiol scavenging by NAC occurred only intracellularly.

2.5 Statistical Analysis

Data were reported as means ± SD. Normality was assessed by the Kolmogorov-Smirnov test. Statistical comparisons were performed by means of the independent-samples Student's *t* test. A value of $p < 0.05$ was considered statistically significant. The data were processed with the statistical package for social sciences (SPSS 16.0, Chicago, IL).

3 Results

3.1 Analysis of Cell Viability

Viability analysis of cultured HaCaT cells was performed using the trypan blue exclusion test following 1 h exposure to CS and aldehydes and was previously reported (Avezov et al. 2014b). A slight, dose-dependent decrease in cell viability was observed ranging from 99.3 ± 0.3 % for cells exposed to 1 cigarette puff to 98.3 ± 1.4 for cells exposed to 3 cigarette puffs (Fig. 1a). After addition of 1 μmol acrolein and 1 h incubation, cell viability was 980 ± 1.4 %, but after addition of 10 μmol, cell viability decreased to 830 ± 1.2.

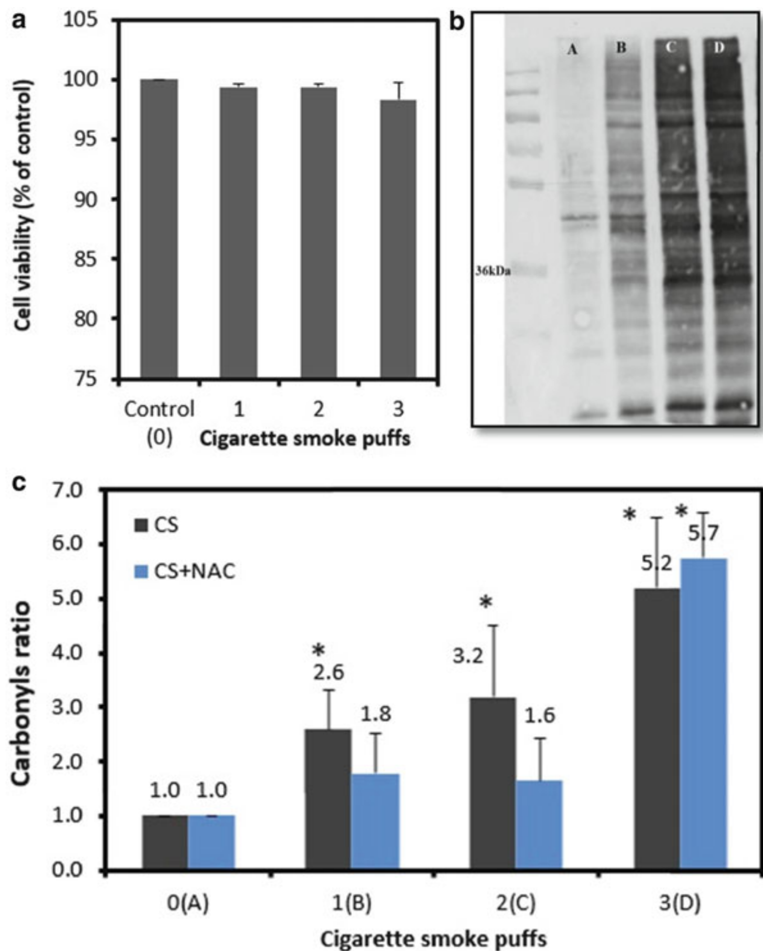

Fig. 1 HaCaT keratinocytes following dose dependent CS exposure. (**a**) Cell viability following exposure to 0 (control) – 3 puffs of CS over a 1 h period; (**b**) Representative WB analysis of total intracellular protein carbonyls. *A*: Untreated control, *B*: After a single puff of CS, *C*: After 2 puffs of CS, *D*: After 3 puffs of CS; (**c**) Intracellular protein carbonyl ratio. Total intracellular carbonylated proteins exposed to CS with and without pre-incubation with NAC. Average densitometric analyses of 3–5 different WB assays of the same experiment (*$p < 0.05$) (Reproduced with permission from: Avezov et al. 2014b)

3.2 Analysis of Intracellular Protein Carbonylation Following CS Exposure

The results of dose-dependent CS induced protein carbonylation (Fig. 1b) in the whole-cell lysates was previously described by our study group (Avezov et al. 2014b). One hour exposure of the HaCaT keratinocyte culture to increasing volumes of CS induced a marked, dose-dependent increase in protein carbonylation. One puff of CS generated an elevation of 2.6 times ($p < 0.01$), 2 puffs induced an elevation of 3.2 times ($p < 0.04$), and 3 puffs of CS increased the carbonyls content by 5.2 times ($p < 0.03$). Cell cultures pre-incubated with NAC (1 mM) for 1 h prior to CS exposure, demonstrated less carbonyl formation in the 1 and 2 CS puff groups, but not in the 3 CS puff group ($p < 0.02$) (Fig. 1c).

In another experiment, cell culture dishes were exposed to 1 CS puff over increasing time intervals. 10 min of incubation generated 1.7 times elevation in protein carbonyls ($p < 0.01$), followed by 2.1 times elevation after 20 min ($p > 0.05$) and 2.6 times elevation after 60 min ($p < 0.01$) (Fig. 2a). One hour preincubation of cell cultures with 1 mM of NAC prior to CS exposure, neutralized part of the carbonyl formation (Fig. 2b).

3.3 Analysis of Intracellular Protein Carbonylation Following Acrolein Exposure

Cell cultures were exposed to 1 μmol acrolein for 20 and 60 min intervals. After 20 min of incubation, protein carbonyl levels elevated by 2.2 times compared with control ($p < 0.01$). After 60 min, protein carbonyls elevated by 2.7 times ($p < 0.05$) (Fig. 3a).

Dose dependent carbonyl accumulation was observed following incubation with 1 and 10 μmol acrolein (equivalent to CS acrolein content from 1 and 10 cigarettes, respectively) over a 1 h period (Avezov et al. 2014b). 1 μmol acrolein addition caused an increase of 2.7 times ($p < 0.04$), while 10 μmol acrolein addition caused a 5 times ($p < 0.01$) increase in intracellular carbonyls. Over a shorter interval of 20 min,

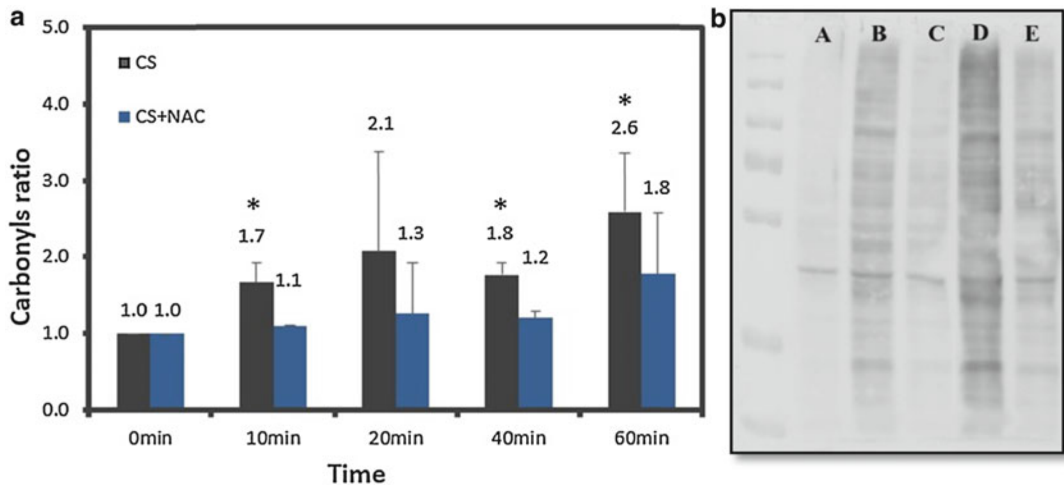

Fig. 2 HaCaT keratinocytes following time dependent CS exposure. (**a**) Intracellular protein carbonyls following exposure to 1 CS puff over 0–60 min, with and without 1 h preincubation with NAC 1 mM. Average densitometric analyses of 3–5 different WB assays of the same experiment; (**b**) A representative WB analysis of total intracellular protein carbonyls. *A*: Untreated control, *B*: After 10 min of exposure to a single puff of CS, *C*: 10 min of exposure to a single puff of CS of cells preincubated with NAC (1 mM), *D*: After 20 min if exposure to a single puff of CS, *E*: After 20 min if exposure to a single puff of CS of cells preincubated with NAC (1 mM) (*$p < 0.05$)

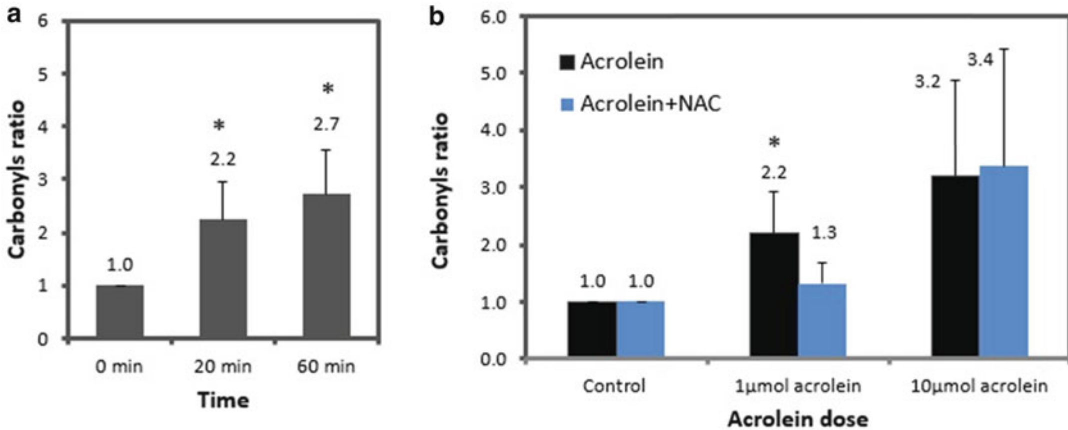

Fig. 3 HaCaT keratinocytes following acrolein exposure. Average densitometric analyses of 3–5 different WB assays of the same experiment. (**a**) Intracellular protein carbonyls following exposure to 1 μmol acrolein for 20 and 60 min; (**b**) Intracellular protein carbonyls following exposure to 1 and 10 μmol acrolein over 20 min interval with and without 1 h preincubation with NAC 1 mM (*$p < 0.05$)

a dose-dependent carbonyl accumulation also was observed. Incubation with 1 and 10 μmol acrolein elevated protein carbonyls by 2.2 ($p < 0.05$) and 3.2 ($p < 0.01$) times, respectively (Fig. 3b). NAC 1 mM 1 h preincubation reversed the damage in the 1 μmol, but not in 10 μmol, group.

4 Discussion

The objectives of the current study were to examine the short time and dose effects of direct CS and acrolein exposure on the intracellular protein carbonylation of keratinocytes. To simulate the influence of cigarette smoking on oral cells, a unique smoking apparatus was used. It allows studying the effects on cell cultures of whole phase CS constituents dissolved in the cell surrounding liquid. A distinct advantage of this method is the use of the same smoke the smokers are exposed to in real life, in contrast to other studies (Park et al. 2012; Kreindler et al. 2005), where CS extract was used, which could likely alter the smoke content. In parallel, the effect of dissolved acrolein was studied in order to isolate its influence and to compare to CS.

In our previous study (Avezov et al. 2014b), it has been established that the effects of whole phase CS applied using the above method is dose-dependent. Even a single puff of CS elevates intracellular carbonyls by 2.6 times. Every additional puff causes a further increase (Fig. 1). In the current study, a short time effect was examined. A single puff of CS was applied to the cells and carbonyl intracellular elevation was observed for a total of 60 min. The results show that as fast as 10 min following CS exposure, an approximate time of smoking a single cigarette, a significant protein carbonylation was observed within the cells. The longer the cells were incubated with CS, the greater was the increase in protein carbonylation. Preincubation with 1 mM NAC partially prevented this elevation, suggesting that NAC thiolic groups were stable and available for this time duration.

Short time effects of pure acrolein exposure were also observed. The cells were exposed to 1 μmol of acrolein over 1 h period. Protein carbonyls accumulated within 20 min of exposure, but continued to rise for 60 min of exposure. Within 20 min of exposure, NAC addition was effective in the 1 μmol but not 10 μmol group. This is due probably to the overload of acrolein, making the addition of 1 mM of NAC insufficient.

As previously mentioned, acrolein is most active at the site of contact; therefore the importance of oral exposure during tobacco products

consumption. Because of its fast degradability in air and soil, no toxic effects through food and environmental exposure have been shown in the general population (Gomes et al. 2002). The situation is different in smokers who are constantly and directly exposed to CS toxic constituents. In the lung tissue of smokers, acrolein is established as a carcinogen due to a pattern of DNA damage in the p53 tumor suppressor gene that resembles the p53 mutations found in lung cancer (Feng et al. 2006). No analogous study has been performed in the oral tissues so far. The current study shows that even a short time and dose exposure is able to cause protein carbonylation, therefore influencing cellular contents. This is particularly important when considering the harm of passive and occasional smoking (Beadsmoore et al. 2007). Furthermore, antioxidant capacity of saliva is reduced by CS aldehydes which interact with thiol rich compounds, such as glutathione (GSH) in the oral fluid. Even a single cigarette is sufficient to impair the protective role of GSH against the noxious biochemical effects of CS (Zappacosta et al. 2002).

On the other hand, continued exposure to additional CS puffs and prolonged incubation time, demonstrate the cumulative nature of CS and acrolein damage. The longer the cells are surrounded with dissolved acrolein, the higher its effect. Sequential smoking of more than one cigarette, contributes to both increased dose and time of acrolein exposure of oral cells. That may be appreciably detrimental in people with reduced salivary flow, a known risk factor for oral pathologies (Porter et al. 2004).

In conclusion, CS and acrolein intracellular protein carbonylation is dose- and time-dependent. Even a short time exposure to CS and its aldehydic constituents can be potentially harmful.

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Diffusion Limitations of the Lung – Comparison of Different Measurement Methods

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In every breath we breathe two graces share – The indraught and the outflow of the air; that is a toil, but this refreshment brings; So marvellous are our life's comminglings. (Johann Wolfgang von Goethe 1819)

Abstract

Pulmonary fibrosis leads to a decrease of oxygen diffusion, in particular during exercise. Bronchial obstruction also could decrease the partial pressure of oxygen (P_aO_2). In this study we investigated the validity of blood gas content, especially P_aO_2 and P_aO_2 affected by hyperventilation (P_aO_{2corr}) and alveolo-arterial oxygen gradient ($P_{A\text{-}a}O_2$) in comparison with the CO diffusion capacity (DL_{CO}) in different lung diseases. A total of 250 subjects were studied (52.3 ± 12.5 year; F/M 40/210), among which there were 162 subjects with different lung disorders and 88 healthy controls. Pearson's correlation coefficients (r) of DL_{CO} with P_aO_2, P_aO_{2corr}, and $P_{A\text{-}a}O_2$ were analyzed in each group. The results show that the diagnostic power of $P_{A\text{-}a}O_2$ against P_aO_{2corr} was equivalent, especially during exercise ($r = -0.89$ and -0.92, respectively). DL_{CO} showed only weak correlations with P_aO_{2corr} and $P_{A\text{-}a}O_2$ ($r = 0.17$ and -0.19, respectively). In conclusion, DL_{CO} shows a better match with blood gas content during exercise than at rest during which it is routinely tested. Thus, the exercise test is advisable. The $P_{A\text{-}a}O_2$ takes into account the level of ventilation, which makes it correlate better with DL_{CO} rather than with blood gas content. The most significant problems in clinical evaluation of blood gas parameters during exercise are the insufficiently defined limits of normal-to-pathological range.

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Keywords
Gas diffusion • Lung • Blood gas content • Cardiopulmonary exercise testing • Oxygen uptake • Alveolar arterial oxygen gradient • Hyperventilation

1 Introduction

Basic prerequisite of any organism is the oxygen supply to all its cells. The central organ of gas transportation is the lung and the blood is a carrier. Oxygenation is achieved by the ventilatory gas exchange and diffusion of gases through the alveolar and capillary walls. The measurement and determination of gas exchange in the lung is therefore a key requirement in the diagnosis of vital functions, especially in obstructive and restrictive lung diseases.

Commonly used is the measurement of the diffusing capacity for CO (DL_{CO}); CO diffusion having the characteristics similar to those of O_2 diffusion. An alternative is a measurement of the results of gas exchange, namely the content of O_2 and CO_2 in arterial or arterialized capillary blood. The diffusion capacity of the lung for gas represents an integral of respiratory function since ventilation, diffusion, and perfusion are included in the measurement.

In general, restrictive lung disease, like fibrosis, results in decreases of oxygen diffusion and arterial oxygen partial pressure (P_aO_2), especially during exercise, compared with obstructive airway diseases which are often associated with a mismatch of ventilation and perfusion. Although both diseases show the key symptom of dyspnea, the cause of dyspnea may be diverse.

Both hyperventilation and hypoventilation affect the pulmonary uptake of O_2 and consequently the P_aO_2: hyperventilation leads to an increase of P_aO_2, so that a malfunction of gas exchange may be underestimated. Therefore, determination of diffusion characteristics through blood gas analysis should take into account the level of ventilation. This integration of ventilation in the assessment of blood gases is enabled by determining the alveolar-arterial oxygen difference ($P_{A\text{-}a}O_2$). It requires not only the measurement of blood gas content but also of the breathing gases O_2 and CO_2 by means of a complex technique. This methodology is always part of a cardiopulmonary exercise testing (CPX).

Another way to calculate the influence of hyperventilation on the P_aO_2 is to assess a decrease in the arterial partial pressure of carbon dioxide (P_aCO_2). The P_aCO_2 is here used to quantify the influence of ventilation; the P_aO_2 can thus be 'corrected' according to the formula: $P_aO_{2corr} = P_aO_2 - 1.66 \times (40\text{-}P_aCO_2)$ (Diekmann and Smidt 1984). The mathematical correction of P_aO_2 is much simpler to perform than the determination of the $P_{A\text{-}a}O_2$. The question arises whether the results of these two measurements are equivalent. Therefore, in the present study we investigated the validity of P_aO_2, P_aO_{2corr}, and $P_{A\text{-}a}O_2$ – all in comparison to DL_{CO} in various lung diseases.

2 Methods

2.1 Participants

The participants were recruited in our occupational outpatient clinic over a period of 5 years and all of them gave signed written consent to use their samples and data. The study was approved by the Internal Medical Review Board. They presented themselves for the diagnosis of work-related diseases and occupational medical examinations. A total of 250 subjects (mean age 52.3 ± 12.5 year; F/M 40/210) were eligible and consecutively included after they had performed CPX with blood gas analysis, DL_{CO}, or both as part of their routine investigation. In addition, medical history, physical examination, spirometry, and body plethysmography were taken in all subjects. We excluded 10 cases due to single missing values. From the remaining

240 individuals, there were: 13 with restrictive lung disorder, defined as VC < lower limit of normal (LLN) (with normal FEV_1/VC, DL_{CO} normal or reduced); 19 with normal VC but DL_{CO} < LLN; 86 with mild or moderate bronchial obstruction (FEV_1/VC < LLN, VC > LLN), 34 subjects with a mixed obstructive/restrictive lung disorder (FEV_1/VC < LLN, VC < LLN), and 88 healthy controls without past or present pulmonary disorders and with normal lung function results. The examinations were performed as part of routine social security screening.

2.2 Lung Function Tests

Lung function and CPX tests were carried out according to the quality criteria of the European Respiratory Society (ERS) and the American Thoracic Society (ATS) (Meyer et al. 2013; Macintyre et al. 2005; Miller et al. 2005; American Thoracic Society 2003). Blood gas analysis at rest was based on the target values of Woitowitz et al. (1969) and the DL_{CO} on those of Cotes et al. (1993). The P_aO_2 during exercise was deemed pathological if it fell below the predicted value by ≥5 mmHg (Meyer et al. 2013). The $P_{A\text{-}a}O_2$ was calculated at rest and under load from the measured values of CPX using the formula: $P_AO_2 = FiO_2 \bullet 713\text{-}(P_ACO_2/$respiratory exchange rate (RER)) (Riley and Cournand 1949). A pathological increase was assumed at a value of >20 mmHg at rest and >35 mmHg during exercise (Meyer et al. 2013; American Thoracic Society 2003).

2.3 Statistical Analysis

Pearson's correlation coefficient (r) was analyzed for DL_{CO} with P_aO_2, DL_{CO} with P_aO_2 after correction of ventilation and DL_{CO} with $P_{A\text{-}a}O_2$, all measured at rest and under load. Correlations of blood gas-dependent parameters (P_aO_2, P_aO_{2corr}, and $P_{A\text{-}a}O_2$) were determined with each other; all calculations were carried out separately for each group of lung diseases.

Cohen's kappa coefficient (Grouven et al. 2007; Thompson and Walter 1988) was calculated to assess the conformity of the values measured with different methods; where '1' indicates a full match, '0' indicates a purely random coincidence, and negative values represent an even lower than a random match. Crosstabs were made to compare the quality of different measurement methods (healthy/pathological assessments). In 39 male subjects, DL_{CO} values were evaluated as based on the level of current hemoglobin concentration corrected (Mottram et al. 1999) and compared with the DL_{CO} of the total cohort. All correlations were calculated according to Pearson (1909), as all variables were interval scaled and normally distributed. Statistical analysis was performed with a commercial SPSS package ver. 19 and 20.

3 Results

The DL_{CO} value (% predicted value) showed in the total cohort only a low correlation of 0.25 ($p < 0.001$) to P_aO_2 at rest and a moderate correlation of 0.57 ($p < 0.001$) to P_aO_2 during exercise (Fig. 1a, b, Table 1). The measurement of $P_{A\text{-}a}O_2$ under load, which takes into account ventilation, showed only a moderate correlation with DL_{CO} of −0.47 ($p < 0.001$) in the total cohort. This correlation remained at a similar level of 0.44 for the 'corrected' P_aO_2 that takes into account P_aCO_2 (Table 1). Higher correlations were found in the first two groups of restrictive lung disease (corresponding with reduced VC or normal VC, but reduced DL_{CO}). Poor correlations in the group with normal lung function values ('healthy lung') can be explained by the closely adjacent individual values (see dense point clouds of this group in Fig. 1a, b).

The P_aO_2 and $P_{A\text{-}a}O_2$ highly correlated with each other at rest and also under load ($r = -0.83$, $r = -0.83$, respectively), which was particularly evident comparing the P_aO_{2corr} and $P_{A\text{-}a}O_2$ with respect to the ventilation values ($r = -0.89$, $r = -0.92$, respectively) (Fig. 2a, b, Table 2). Such high correlations were confirmed by differentiated calculations using crosstabs and kappa values. Kappa values and crosstabs showed a strong concordance, especially under load ($\kappa = 0.69$), particularly within the group of

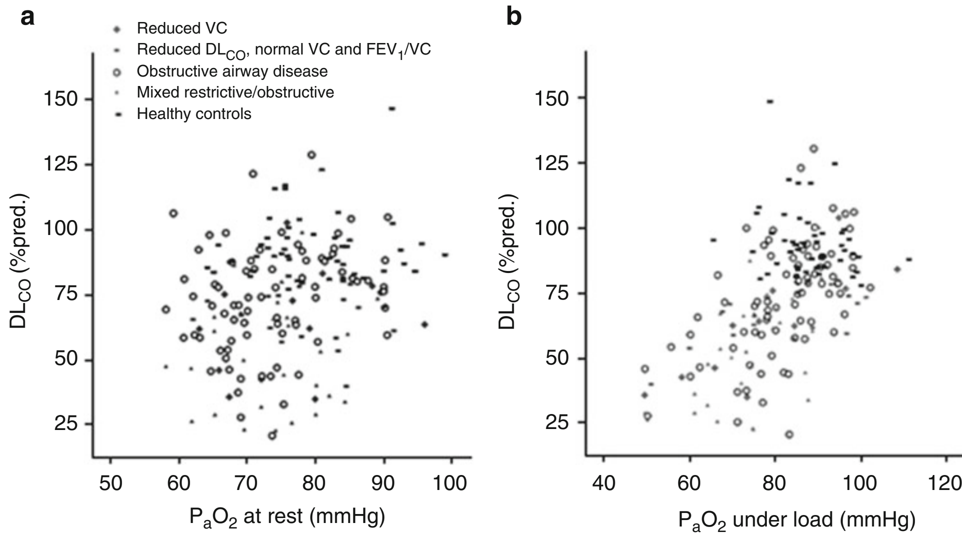

Fig. 1 Correlation between DL_{CO} (%pred.) and P_aO_2 at rest (**a**) and under exercise load (**b**)

Table 1 Correlation coefficients (r) of DL_{CO} with P_aO_2 at rest and P_aO_2 under load, and with $P_{A\text{-}a}O_2$ and P_aO_{2corr} under load

	n	DL_{CO}–P_aO_2 at rest	DL_{CO}–P_aO_2 under load	DL_{CO}–$P_{A\text{-}a}O_2$ under load	DL_{CO}–P_aO_{2corr} under load
All	240	0.25***	0.57***	−0.47***	0.44***
Restrictive lung disease	13	0.30	0.82***	−0.84***	0.74**
Decreased DL_{CO}, normal VC	19	−0.17	0.68**	−0.64**	0.58*
Obstructive airway disease	86	0.24*	0.55***	−0.47***	0.51***
Mixed restrictive/ obstructive	34	0.01	0.58***	−0.41*	0.30
Unobtrusive lung function	88	0.09	−0.23	0.19	−0.34*

*p < 0.05, **p < 0.01, ***p < 0.001

restrictive lung disease ($r = -0.95$, $\kappa = 0.68$). Therefore, $P_{A\text{-}a}O_2$ offers no diagnostic advantage over the corrected P_aO_2 (Table 3), wherein this consideration is essentially dependent on the underlying limits of normal.

When not using the specified correction for load of minus 5 mmHg for the lower limit of P_aO_2 (Meyer et al. 2013), a full match ($\kappa = 1.0$) of the crosstabs for the $P_{A\text{-}a}O_2$ compared with P_aO_{2corr} was present in the group of restrictive lung disease (data not shown). The crosstabs and kappa values for the blood-gas dependent parameters at rest (P_aO_{2corr} and $P_{A\text{-}a}O_2$) with the DL_{CO} showed weak correlations (DL_{CO} and P_aO_{2corr}: $r = 0.17$, $\kappa = 0.10$; DL_{CO} and $P_{A\text{-}a}O_2$: $r = -0.19$, $\kappa = 0.06$) in the total cohort.

Blood gas levels in combination with their corresponding parameters from the exercise test showed a moderate correlation to DL_{CO} and – according to the kappa value – low dependence in the clinical assessment (DL_{CO} and P_aO_{2corr} under load: $r = 0.44$, $\kappa = 0.22$; DL_{CO} and $P_{A\text{-}a}O_2$ under load: $r = -0.47$, $\kappa = 0.23$, see Table 4). Similarities were mainly in the group of persons with restrictive lung disease; even there, significant correlations were present when

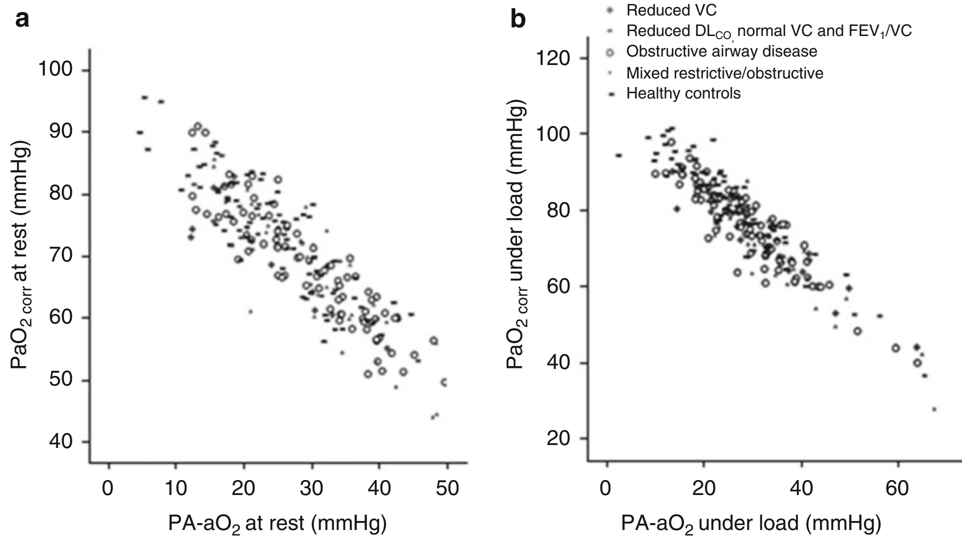

Fig. 2 Correlation of P_aO_{2corr} and $P_{A\text{-}a}O_2$ at rest (**a**) and under exercise load (**b**)

Table 2 Correlation coefficients (r) of $P_{A\text{-}a}O_2$ with P_aO_{2corr} at rest and under load

	n	$P_{A\text{-}a}O_2$–P_aO_{2corr} at rest	$P_{A\text{-}a}O_2$–P_aO_{2corr} under load
All	240	−0.89***	−0.92***
Restrictive lung disease	13	−0.85***	−0.95***
Decreased DL_{CO}, normal VC	19	−0.86***	−0.98***
Obstructive airway disease	86	−0.91***	−0.91***
Mixed restrictive/obstructive	34	−0.90***	−0.91***
Unobtrusive lung function	88	−0.87***	−0.90***

***$p < 0.001$

Table 3 Kappa values (κ) for comparison of P_aO_{2corr} with $P_{A\text{-}a}O_2$ under load: **A** – in the total cohort and **B** – in the restrictive lung disease group

A		$P_{A\text{-}a}O_2$ under load		
P_aO_{2corr} under load		Pathological (>35 mmHg)	Normal	Total
	Pathological (<LLN–5 mmHg)	35	7	42
	Normal	16	169	185
	Total	51	176	227[a]
$\kappa = 0.69$				
B		$P_{A\text{-}a}O_2$ under load		
P_aO_{2corr} under load		Pathological (>35 mmHg)	Normal	Total
	Pathological (<LLN–5 mmHg)	4	2	6
	Normal	0	7	7
	Total	4	9	13
$\kappa = 0.68$				

[a]For 13 subjects, there were no values at exercise, therefore they are not rated, this implies the difference to 240

Table 4 Kappa values (κ) for comparison of DL_{CO} at rest with: **A** – P_aO_{2corr} under load and **B** – $P_{a\text{-}a}O_2$ under load

A		DL_{CO} at rest		
P_aO_{2corr} under load		Pathological (<LLN)	Normal	Total
	Pathological (<LLN–5 mmHg)	31	9	40
	Normal	66	82	148
	Total	97	91	188[a]
$\kappa = 0.22$				

B		DL_{CO} at rest		
$P_{A\text{-}a}O_2$ under load		Pathological (<LLN)	Normal	Total
	Pathological (>35 mmHg)	36	13	49
	Normal	61	78	139
	Total	97	91	188[a]
$\kappa = 0.23$				

[a]For 52 subjects there were no values for DL_{CO} or exercise test, this implies the difference to 240

the blood gas-dependent values were obtained under load (DL_{CO}–P_aO_{2corr} load: $r = 0.74$, $\kappa = 0.24$; DL_{CO}–$P_{A\text{-}a}O_2$ under load: $r = -0.84$, $\kappa = 0.41$). In the other groups (obstructive airways disease; mixed restrictive/obstructive disorders; unobtrusive lung function), the similarities were rather weak (data not shown).

As a supplement, the difference between the DL_{CO} value corrected to the current level of hemoglobin and the otherwise underlying DL_{CO} value was calculated in 39 male patients (with assumption of a hemoglobin level of 14.6 g/dL). There was a small deviation in the mean DL_{CO} of 3.0 ± 2.2 %. This outpatient study, with the exclusion of severely ill people, showed the influence of the hemoglobin level to be of little relevance.

4 Discussion

Blood gas analysis and DL_{CO} are the most important diagnostic steps in the assessment of pulmonary gas exchange in routine diagnostics. However, both methods are not always available and their results are affected differently by the respective pulmonary disease and hypo- or hyperventilation. The determination of the alveolar-arterial oxygen difference ($P_{A\text{-}a}O_2$) allows the inclusion of ventilation in the assessment of blood gases. This, in turn, requires not only the blood gas analysis, but also the determination of the exchange of respiratory gases O_2 and CO_2 by means of a complex measuring equipment. Such equipment is part of a cardiopulmonary exercise testing and the method is thus used frequently in the context of cardiopulmonary exercise tests. The influence that ventilation exerts on the P_aO_2 can be assessed by calculating the exhaled CO_2 and thus the ‘correction’ of P_aO_2 can be made. The P_aCO_2 is used to quantify the influence of ventilation. In practice, the corrected P_aO_2 is attained with the results of blood gas analysis in combination with a simple ‘correction formula’ (Diekmann and Smidt 1984). Furthermore, we wanted to verify whether the blood gas values allow for the identification of various lung gas exchange disorders, such as – based on an entirely different principle of measurement – determination of diffusion of CO in the lung (DL_{CO}). It should be noted that the DL_{CO} is to be determined only at rest.

The DL_{CO} is a result of two measurements during a single-breath method (Hughes and Pride 2001): the diffusion gradient at the alveolar membrane and the ventilated alveolar volume. Both result from the measurements of volume, gas concentrations, and calculations. The assumption that DL_{CO} correction using the alveolar volume (DL_{CO}/VA) leads to a more accurate determination of lung diffusion capacity cannot be confirmed by recent publications, since the change of the quotient is not constant with the change of alveolar volume (Hughes and Pride

2012). Therefore, just DL_{CO} and not the DL_{CO}/VA ratio was considered in the context of the present work. The DL_{CO} is considered the gold standard to verify lung diffusion disorders, regardless of their genesis. A closer look at our results reveals that this may apply only for restrictive lung diseases, at least in comparison with blood gas analysis, even after correction of ventilation. A decrease in P_aO_2 found in blood gas analysis also points to this disease, but the severity of gas exchange impairment could be underestimated if hyperventilation is not observed. Therefore, the arithmetical correction of ventilation can also be useful here.

In obstructive airway disease, in 45 % of cases, the CO diffusion disorder cannot be confirmed by blood gas analysis and $P_{A\text{-}a}O_2$ during exercise. In our opinion, the DL_{CO} also appears negatively affected by inhomogeneity of ventilation and perfusion resulting in gas exchange disorders even at rest. This inhomogeneity is known, in particular, for obstructive lung diseases. Only exercise tests seem to provide a better differentiation of a fixed diffusion disorder. Schwarz et al. (1999) also concluded that the $P_{A\text{-}a}O_2$ determined by CPX is a more sensitive parameter, compared with P_aO_2, in the evaluation of gas exchange disorders. The authors also found only a weak correlation of DL_{CO} to $P_{A\text{-}a}O_2$. Both parameters would have a better match if one would measure not only P_aO_2, but also DL_{CO} under load. This would counterbalance the ventilation-perfusion inequality. This inhomogeneity in obstructive lung disease appears of less importance in restrictive lung diseases. DL_{CO} measurements in the loading condition would give a truer assessment of the factual gas diffusion – however, this is not yet available. Furthermore, inhomogeneity of lung perfusion and ventilation under load would be reduced and the entire system of gas exchange would be tested at load limit.

At present, DL_{CO} and blood gas content measurements do not provide comparable values enabling their clinical evaluation. To detect malfunctions in the system, the blood gas content under load, determined with a correction of P_aO_2 in rapport with the level of ventilation, has the best explanatory power for clinical assessment. The $P_{A\text{-}a}O_2$ has a similar power, but there are no reliable set point-values, which complicates the clinical evaluation. Thus, $P_{A\text{-}a}O_2$ shows no significant advantage over the P_aO_2. Nevertheless, one should - as also others report (Schwarz et al. 1999) – use the parameters associated with history, clinical, laboratory values, and imaging techniques. Exercise testing enhances the evaluation of severity, prognosis, and treatment monitoring (Meyer et al. 2013).

The collected absolute values – in particular, the blood gas parameters derived there from $P_{A\text{-}a}O_2$ and P_aO_{2corr} – are well comparable and highly correlated, so that the explanatory power of the $P_{A\text{-}a}O_2$ against the P_aO_{2corr} seems diagnostically equivalent. However, given the need to use the CPX system to determine ventilation and $P_{A\text{-}a}O_2$ or the above-mentioned calculations to obtain P_aO_{2corr}, using the P_aCO_2, there is a small advantage compared with the determination of P_aO_2 alone. The main challenge consists of setting a demarcation line between normal and pathological values. This is by far only vaguely defined for $P_{A\text{-}a}O_2$ with a limit of 35 mmHg across all age groups and all load levels. The assessment of blood gases also shows discrepancies. The generally assumed limit of 5 mmHg below the normal value is questioned as it may be age- and exercise-dependent.

For the present study, it is essential to note that X-ray images of the subjects investigated were not always present. It was assumed that relevant changes would be reflected in pathological lung function values. It should also be noted that the maximum workload was not defined and was not included in the analysis. The subjects achieved their individual maximum wattage depending on gender, height, weight, age, fitness level, and an existing lung disease. The increase in wattage per minute was selected depending on the expected overall performance and took place after 8–12 min (Preisser and Ochmann 2011). Accordingly, these factors were highly variable. The aim of the study was to capture the effect of blood circulation and pulmonary ventilation as prominently as possible; therefore, the endpoint of maximum workload was selected.

5 Conclusions

In general, only a few conclusions about the existence of a gas exchange disorder can be drawn from the blood gas analysis at rest. The DL_{CO} also is determined only at rest, but shows a slightly better match with the results of the blood gas content during exercise than that at rest. The exercise testing is thus desirable.

Exercise testing is becoming increasingly important in the evaluation of disease severity, prognosis, and therapy monitoring. The inhomogeneity of perfusion and ventilation, influencing the P_aO_2, can be revealed through the exercise test, not only for obstructive airway diseases but also in healthy subjects (Meyer et al. 2013). The exercise test should include the determination of blood gases, and in the case of cardiopulmonary exercise tests, also the alveolar-arterial oxygen difference. The $P_{A\text{-}a}O_2$ takes into account the level of ventilation, thus it probably has a better correlation with DL_{CO} compared with the blood gas analysis.

In restrictive lung disease, all three parameters are comparably suitable to detect the gas exchange disorder. In obstructive airway disease, DL_{CO} seems affected by other pathophysiological aspects; thus there is only a moderate correlation with the blood gas-based parameters. The blood gas analysis at rest can lead to false-negative results, especially in case of restrictive lung disease. The DL_{CO} indicates more likely false-positive results, especially for obstructive airway diseases.

Changes in $P_{A\text{-}a}O_2$ and P_aO_2 during exercise are highly comparable in patients with restrictive lung disease. An exercise test with the determination of blood gases seems to be diagnostically adequate in these cases, at least for the evaluation of gas exchange disorders.

The 'correction' formula of Diekmann and Smidt (1984) is also applicable for the blood gas content in the exercise load condition, but gives no advantage over the P_aO_2 in the assessment of different lung diseases. However, clinical classification as 'normal' or 'pathological' shows only a moderate difference in $P_{A\text{-}a}O_2$ and in ventilation corrected P_aO_2. The most significant problem in the clinical application of the blood gas parameters at exercise and of $P_{A\text{-}a}O_2$ stems from the lack of clearly defined normal-to- pathological range, in particular, for the $P_{A\text{-}a}O_2$, where validated reference values are needed.

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Respiratory Infections in Travelers Returning from the Tropics

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Abstract

Respiratory tract infections (RTIs), beside diarrheas, skin lesions, and fevers of unknown origin, are one of the most common health problems acquired by travelers going to tropical and subtropical countries. Visitors to African, Asian, or South American destinations, typically characterized by harsh environmental conditions and poor sanitation standards, are at risk of exposure to a large number of pathogens causing infectious diseases. The infections are transmitted from contaminated food and water, through the air, direct contact, or by insects. The main modes of RTIs transmission include droplet infection and direct contact. The clinical spectrum of RTIs in travelers is broad, from upper respiratory tract infections, pharyngitis, bronchitis, pneumonia, to influenza-like illness. The spectrum of microbial agents causing respiratory infections include numerous viruses and bacteria, rarely fungi, and parasites. Most travelers complain of mild infections, only a small minority seek medical assistance and report to health care facilities. Because of the risk of importing pathogens into Europe or North America and transferring them onto the local population, it is important to present the scale of the problem in relation to rapid development of tourism industry and an increasing number of intercontinental journeys. The aim of the study

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was to discuss the occurrence of travel-related respiratory infections among representatives of temperate climate traveling to and returning from the tropics.

Keywords
Respiratory infections • Travelers • Tropics

1 Introduction

The number of travelers from developed European and North American countries to regions lying in a hot climate zone is growing every year. Over the last few decades, the world has become a global village. Nowadays, everybody travels: small children, the elderly, pregnant women, the disabled, and even the chronically ill. People travel for various reasons, e.g. business, research work, tourism, but the majority of travelers are holiday-makers. The risk of acquiring an infection during long-distance travel depends on a number of factors, including the degree of endemicity in a visited part of the world, health condition of a traveler (proper functioning of the immune and thermoregulatory system, or history of chronic diseases), undertaking appropriate measures of health prevention, the length of stay, or types of activities to be undertaken in the destination country (Korzeniewski 2014).

Visitors to areas characterized by harsh climatic conditions and low standards of sanitation run the risk of exposure to vector-borne, food and water-borne and respiratory pathogens, which are rarely found in the temperate climate zone (Leder et al. 2013). Some travelers from industrialized to developing countries suffer health problems which are directly related to foreign travel. A vast majority of travelers complain of mild illnesses and only a small minority seek medical assistance and report to health care facilities. Travelers most commonly complain of diarrheas, skin lesions, fevers of unknown origin, and respiratory infections (Harvey et al. 2013).

The aim of this article is to discuss the occurrence of travel-related respiratory infections among representatives of temperate climate traveling to and returning from the tropics.

2 Epidemiology of Respiratory Tract Infections Among Travelers in the Tropics

About 50 million people travel each year from industrialized countries to tropical or subtropical destinations (Herbinger et al. 2012). Travel to the tropics carries a high risk of morbidity, estimated at 20–70 % (Stienlauf et al. 2005). Worldwide, 8 % of travelers to developing countries report becoming ill enough to seek professional health care during or after travel (Harvey et al. 2013).

Respiratory tract infections (RTIs) are one of the most common travel-related health problems. The significance of RTIs in travelers stems from their high frequency and the year-round presence of pathogens, both the cosmopolitan pathogens and the less-known, yet potentially life-threatening ones (Meltzer and Schwartz 2009). The types of RTIs affecting travelers are usually similar to those observed in the local population, exotic cases are considered a rare event, and most cases are caused by cosmopolitan pathogens (Matteelli et al. 2005). The clinical spectrum of RTIs in travelers is broad. In the group of 1,719 international travelers returning with respiratory infections (7.8 % of all studied group), the main clinical presentations were non-specific upper RTIs (diagnosed in 47 % of the patients), bronchitis (20 %), pneumonia (13 %), pharyngitis (13 %), and influenza-like illnesses (5 %) (Leder et al. 2003). Among 540 Italian patients hospitalized with a history of travel and fever, RTIs were diagnosed in 40 patients (7 % of the

febrile patients) and the most common RTIs were pneumonia (35 %) and tuberculosis (15 %) (Matteelli et al. 2005). The spectrum of microbial agents causing RTIs had been previously described and include numerous viruses (influenza, parainfluenza, respiratory syncytial virus, metapneumovirus, adenovirus, rhinovirus, and coronavirus) and some bacteria (*Streptococcus* sp., *Mycoplasma pneumoniae*, *Legionella pneumophila*) (Pavia 2011).

The most common comprehensive registry of ill travelers returning from the tropics is the multinational GeoSentinel Surveillance Network. During September 1997–December 2011, the data were collected on 141,789 patients with confirmed or probable travel-related diagnoses. The most common reasons for travel were tourism (38 %), missionary/volunteer/research/aid work (24 %), visiting friends and relatives (17 %), and business (15 %). The most common regions of exposure were Sub-Saharan Africa (23 %), Central America (15 %), and South America (12 %). Of the 1,002 diagnoses in the respiratory grouping, 70 % were accounted for by five diagnoses: upper respiratory tract infection (27 %), acute bronchitis (18 %), acute sinusitis (11 %), bacterial pneumonia (lobar) (8 %), and asthma (6 %) (Harvey et al. 2013). In another GeoSentinel study on febrile patients, RTIs accounted for 14 % of all cases (Wilson et al. 2007). In the study of Freedman et al. (2006) in the same base, RTIs accounted for 77/1,000 travelers. Rack et al. (2005) have estimated the respiratory infections incidence among German travelers at 13.8 %, whereas among nearly 5,000 Scottish travelers at 16.8 % (Redman et al. 2006). In all these surveys, RTIs were second to diarrhea as the most prevalent health event during travel. In the study of Ansart et al. (2005), RTIs concerned 11.5 % patients coming from tropical countries. They mainly consisted of pulmonary infections, influenza-like illnesses, and ear, nose, and throat infections. The causes of pneumonia are multiple and include bacterial, viral, and fungal infections and also eosinophilic pneumonitis (related to the invasive stage of various helminthic infections) (Ansart et al. 2004).

Most cases of RTIs in travelers are upper respiratory tract infections (URTIs), which are mild and therefore unreported. The most common pathogens of URTIs are viruses, whose transmission and spectrum is similar in travelers and in the general population, with droplet infection and close contact accounting for most cases (Meltzer and Schwartz 2009). Camps et al. (2008) have diagnosed at least one virus from 56 % of travelers with fever and respiratory symptoms. The most frequent viruses detected were influenza virus (38 %), rhinovirus (23 %), adenovirus (9 %), and respiratory syncytial virus (9 %). Similar results were reported in German travelers, where again influenza was the most frequent isolate (Luna et al. 2007). In Swiss travelers, Mutsch et al. (2005) have established an incidence rate of influenza of 1 case/100 travel months, with 2.8 % overall incidence and 12.8 % among travelers with a history of febrile illness. Askling et al. (2009) studied 1,432 febrile travelers from Sweden who had returned from the tropics. For 21 % of the 115 patients with fever of unknown origin, serologic analysis showed that influenza was the major cause. People at risk of influenza include not only typical tropical travelers but also leisure and business travelers to countries in the temperate zone (including travel within their own country), where influenza is in season, regardless of whether the traveler falls into a usual risk group (Sato et al. 2000). The risk of travelers encountering influenza virus depends both on the travel destination and the time of year. In the temperate regions of the northern hemisphere (North America, Japan, or Europe), most influenza activity occurs from November through April. In the temperate regions of the southern hemisphere (Australia and New Zealand), most influenza activity occurs from April through October. In the tropics, influenza virus circulates at low levels year-round (Harper et al. 2005). Traveling presents unique opportunities for close contact with other travelers, many of whom may harbor influenza and other respiratory pathogens (Freedman and Leder 2005). Cruise ships, with their closed environments and large numbers of passengers from different countries, have been shown to present unusually high risk situations for viral

transmission. Attack rates for influenza-like illness (ILI) have ranged from 17 to 37 % in reported outbreaks (Brotherton et al. 2003). In practice, laboratory confirmation of influenza is performed relatively infrequently, it probably accounts for at least 5–6 % of respiratory illnesses reported in travelers (Leder et al. 2003). Other data support a suspicion that influenza may be the most common vaccine-preventable disease in travelers (Steffen et al. 2008). The largest annual transit of persons in the world is probably the Hajj. In a large survey among Hajj pilgrims in 2003, respiratory infections accounted for 40 % of all reported illnesses, and influenza virus accounted for the half of the isolated respiratory viruses (Balkhy et al. 2004). Because influenza is highly contagious and has a short incubation period, travel contributes considerably to the rapid spread of the virus (Hollingsworth et al. 2007). The World Health Organization (WHO) estimates that 5–15 % of the world population is affected by seasonal influenza viruses annually (Hill 2000). In Canada, the Committee to Advise on Tropical Medicine and Travel recommends influenza vaccination to all healthy travelers. WHO recommends annual vaccination only for travelers who are at high risk for complications of influenza. In the Netherlands, as in other European countries, influenza vaccination is already recommended for these risk groups, irrespective of travel (Belderok et al. 2013).

The spectrum of travel-related etiological agents of lower respiratory tract infections (LRTIs) depends on the region of the world. In Europe and North America the most common pathogen is *Legionella*, in Latin America *Histoplasma*, in Africa *Schistosoma*, and in Southeast Asia geohelminths. Legionellosis typically affects luxury tourism. Most cases are reported after travel to developed countries in Europe (Spain, France, and Italy) or North America. A specific form of travel that is frequently associated with legionellosis outbreaks is ship cruising (Meltzer and Schwartz 2009). In the European Union, the case fatality rate in travel-related legionellosis ranges between 3.8 and 5.6 % (Ricketts et al. 2006). One of the pathogens which is most actively imported to developed countries, especially by immigrants, is *Mycobacterium tuberculosis*. The etiological factor of tuberculosis (TB) is easily transmitted via aerolized droplets in the crowded environments of aircrafts and airports, although outbreak investigations have found that the true infection rate under these conditions is quite low (Meltzer and Schwartz 2009). The risk of TB in tourists, especially in short-term travel, is not clearly established. Such cases were rarely reported to the GeoSentinel system, and indeed the odds ratio for TB was 66.7 in favor of immigrants and visiting friends and relatives (VFRs) as opposed to other tourists (Leder et al. 2006). Similar results are reported from France and Italy, where TB is essentially restricted to immigrants and VFRs, mostly from Africa (Ansart et al. 2005; Matteelli et al. 2005). The significance of TB as a travel-related disease is debated and still not fully recognized. TB has a specific importance among respiratory infections because it can spread through respiratory contact and requires strict isolation conditions in hospitals, and because there is an important threat of resistant cases (Matteelli et al. 2005). Although the prevalence of TB has greatly decreased in the temperate and developed counties of Western Europe, North America, Australia, and Japan, it remains a major disease burden in tropical and developing countries (Freeman et al. 2010; Corbett et al. 2003). Consequently, travelers and expatriates from low-prevalence nations who travel to or live in high prevalence nations may become infected with TB (Cobelens et al. 2000). There is still debate about the risk for latent tuberculosis infection (LTBI) that results from long-term travel (Toovey et al. 2007). Cobelens et al. (2000) suggested that the risk of acquiring LTBI to travelers is similar to that of the general population in the destination country. According to Rieder (2001), many apparent latent TB infections in travelers from low-incidence to high-incidence countries may be due to false positive tuberculin skin tests (TSTs) in this otherwise low-prevalence population. Pseudoepidemics of TST conversions in military populations have been reported in relation to travel (Mancuso et al. 2008).

Risk factors for the acquisition of RTIs during international travel are not clearly identified,

e.g. age, gender, trip duration, or reason for travel as a predictor for developing respiratory infections. O'Brien et al. (2001) reported a five times higher risk of pneumonia among travelers >40 years of age. In evaluating returning travelers, the first issue is to establish by a thorough medical interview whether respiratory complaints are indeed temporally associated with travel. The majority of patients presenting with post-travel respiratory symptoms fall into one of two major categories, an acute febrile diseases with respiratory signs or protracted post-travel respiratory symptoms (Meltzer and Schwartz 2009).

3 Post-travel Respiratory Symptoms in Returning Travelers

Respiratory tract infections are a significant cause of health problems, accounting for 7–11 % of consultations in returning travelers (Jaureguiberry et al. 2011; Freedman et al. 2006). The prevalence of RTIs is invariably higher in travelers presenting with fever, as respiratory infections account for 14–24 % of the etiologies of fever (Wilson et al. 2007). RTIs are the second most common cause of illness in travelers and of fever in returning travelers (O'Brien et al. 2001). Post-travel respiratory infections were reported among 25 % Israeli (Winer and Alkan 2002) and 26 % American travelers (Hill 2000). RTIs were also a reason of hospitalization among 24 % Australian febrile patients returning from the tropics (O'Brien et al. 2001). Pneumonia still accounts for many hospital admissions due to post-travel respiratory illnesses (Stienlauf et al. 2005). Most of the major causes of pneumonia have a global distribution. *Streptococcus pneumoniae*, *Hemophilus influenzae*, and *Staphylococcus aureus* are the dominant pneumonia isolates in developing countries. Some agents of travel-related pneumonia have a global distribution, but are reported infrequently. Q fever, e.g., is rarely diagnosed in travelers, and in fact has more often been associated with a non-specific febrile illness than with RTI. The majority of travel-related Q fever is reported from Africa (Meltzer and Schwartz 2009). Some agents of travel-related pneumonia are rare in most developed countries and may, and thus may be missed by physicians if the travel itinerary is not taken into account. These include some helminthic, bacterial, and fungal respiratory infections (Meltzer and Schwartz 2009). In a study of Ansart et al. (2004), most cases of pneumonia were bacterial, with *S. pneumoniae*, *Mycoplasma*, and *Legionella*, but some etiologies included dengue, leptospirosis, tuberculosis, histoplasmosis, schistosomiasis, and Q fever. The majority of travelers with pneumonia will not differ from routine cases of community-acquired pneumonia (CAP). Yet increased exposure to hotels requires heightened alertness to the possibility of legionellosis. Travelers to East and Southeast Asia may be exposed to *Burkholderia pseudomallei*, the causative agent of melioidosis (Meltzer and Schwartz 2009). Severe pneumonia can also be associated with viral infections. Primary influenza pneumonia is, in fact, underreported, but during pandemic years it has accounted for 18 % of all influenza-associated pneumonia (Rothberg et al. 2008). Other rarely reported viral pneumonias in travelers include SARS and hantavirus pulmonary syndrome. Active tuberculosis is rarely diagnosed in returning travelers. Thus, when a traveler presents with fever and respiratory symptoms in the immediate post-travel period, he is very unlikely to be diagnosed with pulmonary TB (Meltzer and Schwartz 2009). Also, tropical diseases are not the leading cause of consultation in travelers from industrialized countries returning from the tropics. Immigrants from Africa, Asia, and South America are the travelers most at risk of common tropical diseases (Ansart et al. 2005).

It is important to remember that respiratory symptoms are not rare in systemic febrile illnesses that are not commonly associated with the respiratory tract. In malaria, respiratory symptoms occur in up to half of the patients and are not limited to cases with acute respiratory distress syndrome (Anstey et al. 2002). Similarly, cough is not rare in enteric fever and leptospirosis, which are sometimes initially mistaken for RTIs. Among patients with URTIs, fever and

cough with an acute onset are the definition of influenza-like illness. The patterns of influenza seasonality in travel medicine reflect the transmission pattern in the destination countries. It should be remembered that in many tropical countries influenza transmission is continuous rather than seasonal (Lowen et al. 2008).

Acute bacterial infections causing RTIs are usually accompanied by eosinopenia. Pneumonia with eosinophilia can be attributed to helminthic infections. Schistosomiasis manifests acutely as a combination of fever and respiratory symptoms, either with or without pulmonary infiltrates. Similarly, geohelminths (*Ascaris*, *Ancylostoma*/*Necator*, or *Strongyloides*) can cause an acute febrile episode with cough, again with or without lung infiltrates (Meltzer and Schwartz 2009). Respiratory symptoms in a febrile traveler should suggest the presence of common respiratory pathogens such as *S. pneumoniae,* influenza and other respiratory viruses, mycoplasma, and *Legionella pneumophila*. *L. pneumophila* infection can be acquired by travelers in spas, on cruise ships and in hotels (Habib and Behrens 2000). The presence of fever, pneumonia, and hepatitis should prompt consideration of Q fever (caused by *Coxiella burnetii* and associated with animal exposure) (Ryan et al. 2002). One in three febrile travelers is diagnosed with malaria. Therefore, it is important that during the diagnostic process, some consideration should also be given to this disease entity. In travelers returning from tropical or subtropical countries with a non-specific febrile illness it is necessary to perform the following tests: blood test by light microscopy, CBC with differential, AST, ALT, urinalysis, a chest X-ray, examination of stool specimens for parasites, tests for specific disease entities (e.g. HIV). Blood, urine and fecal cultures should also be considered. Another element which may turn useful during the diagnostic process is the incubation period (e.g., the onset of fever more than 3 weeks after returning from a journey virtually excludes the diagnosis of viral hemorrhagic fevers). Most illnesses manifest after a few days/weeks' incubation period. However, some illnesses (tuberculosis, schistosomiasis) have the incubation period of 6 months or more; hence, it is necessary to take detailed history from patients returning from the tropics (Korzeniewski 2014).

4 Summary

Between 20 and 70 % of the people who travel from the industrialized countries to developing world each year experience a health problem associated with their journey. Respiratory tract infections (RTIs) are one of the most common travel-related health problems. The main modes of RTIs transmission include droplet infection and direct contact. The type of RTIs affecting people traveling to, and returning from, the tropics are usually similar to those observed in local populations. Tropical cases are considered a rare event (the main source of tropical illnesses are immigrants from Africa, Asia, and South America). The clinical spectrum of RTIs in travelers is broad, from upper respiratory tract infections (URTIs), pharyngitis, bronchitis, pneumonia to influenza-like illness. The spectrum of microbial agents causing RTIs include numerous viruses and bacteria, rarely fungi and parasites. Most cases of respiratory infections in travelers are URTIs, which are mild and therefore unreported. Traveling presents unique opportunities for close contact with other travelers, many of whom may harbor respiratory pathogens. Excellent examples are cruise ships and Hajj (pilgrimage), with their close environments, large number of passengers/pilgrims, and high risk of RTIs transmission. The pathogen typically affecting luxury tourism (cruise ships, hotels) is *Legionella*, whereas among Hajj pilgrims the most common reported respiratory pathogens are viruses. Respiratory symptoms are not rare among systemic febrile illnesses that are not commonly associated with the respiratory tract, e.g., one in three travelers with fever of unknown origin is diagnosed with malaria. Similarly, cough is not uncommon in enteric fever and leptospirosis, which can initially be mistaken for RTIs. Fever and cough with an acute onset are typical symptoms of influenza-like illness. An acute fever episode with cough can be also observed in geohelminths infections, fever and pneumonia in

Q fever. Travelers returning from the tropics who report to a health care facility with symptoms of infection need to undergo a thorough clinical examination. It is also necessary that a detailed history be taken from such patients, to determine the incubation period and risk factors, as that facilitates targeted diagnosis and further treatment.

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Relative Risk of Lung Obstruction in Relation to PM_{10} Concentration as assessed by Pulmonary Function Tests

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Abstract

Epidemiological studies show that long-term exposure to air pollution may increase the relative risk of obstructive lung diseases such as COPD or asthma. The risk of increased obstruction is higher among residents living in close proximity to high traffic routes where there are high concentrations of PM_{10}. The present study consists of two parts: the measurement of the concentration of air pollutants and of pulmonary function in selected groups of people. The study was conducted in Warsaw, Poland, in seven localizations with typical urban canyon characteristics and roads with high traffic. The control group consisted of people living in other regions of Poland with a significantly lower ($p < 0.05$) concentration of air pollutants. The study was performed in the years 2008–2012. The incidence of obstructive lung disease was determined according to the GOLD guidelines. The study subjects were all non-smokers. The relative risk of disease took into account different exposure times to air pollutants. The findings indicate that an increase in PM_{10} concentration by each 10 μg/m^3 caused an increase in the relative risk of lung obstruction by a factor of 1.27, 1.24, and 1.19 for the residence period in the vicinity to heavy traffic city roads for 20, 30, and 40 years, respectively as compared with the residence of rural unpolluted areas. A decrease in the number of people with lung obstruction with the length of residence actually indicates that people exposed to high concentrations of PM_{10} become affected by lung obstruction at a lower age. The study shows a positive relative risk of lung obstruction due to an exposure to high PM_{10} emission.

Keywords

Air pollution • Asthma • Lung obstruction • PM_{10} • Relative risk

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1 Introduction

The air pollution contributes to 8 million deaths annually, which constitutes 15 % of deaths of all causes over the world. Exposure to ambient air pollution causes an increase in morbidity and mortality of a variety of diseases, including respiratory diseases such as asthma or COPD, cardiovascular diseases (Arena et al. 2006; Wong et al. 2002; Pope et al. 2004). According to Global Burden of Disease Study (Murray et al. 2012), chronic obstructive pulmonary disease (COPD), is the third most common cause of death worldwide, and comes in nine place in a rating based on the disability life adjusted years (DALY) indicator. Asthma is not as serious as COPD, taking into account the number of deaths and lost DALY years, and is not listed in the first 30 disorders in the GBDS ranking (Lozano et al. 2012; Murray et al. 2012). COPD and asthma are characterized by a decrease in airway flow, which is usually assessed by respiratory function tests. The severity and progression of COPD is determined by forced expiratory flow in 1 s (FEV_1), expressed as a percentage of predicted value (Vestbo et al. 2013).

COPD reduces a person's quality of life and imposes a substantial economic burden on society due to decreased productivity, absenteeism, and health care costs. It is estimated that the 2008 indirect costs of COPD and asthma-related premature mortality were as high as 18 billion USD in the US (National Heart, Lung, and Blood Institute 2012). In the EU, as much as 6 % of the healthcare budget is dedicated to treatment of respiratory disorders, out of which 56 %, i.e., 36.8 billion EUR are COPD-related costs (Rabe et al. 2007).

Although the most significant risk factor of COPD and asthma is tobacco smoking, the disease affects non-smokers as well (Kohansal et al. 2009). It is estimated that non-smokers constitute 25–45 % of all COPD cases (Salvi and Barnes 2009). Therefore, it is important that the epidemiological studies on the influence on human health of air suspended particles took into account a non-smoking population as well.

The short-term studies on the influence on the human health of the air PM_{10} concentration have clearly shown an increase in hospital admissions related to asthma or COPD, including increased mortality caused by these diseases (Peacock et al. 2011; Schwartz 1996). The long-term studies, albeit less often conducted, also show an increased incidence and intensification of COPD symptoms in persons exposed to increased PM_{10} concentration (Gan et al. 2013; Andersen et al. 2011). One of the methods for the relative risk assessment of the incidence of lung obstruction in a population is to study a correlation between increased concentration of pollutants and decreased respiratory indices. For instance, a study on air pollution and lung diseases in adults (SAPALDIA) showed a fall in FEV_1 of 1.6 %, and FVC of 3.4 % when PM_{10} increased by 10 μg/m^3 in relation to the concentration of this particular pollutant in the area inhabited by a control group (Ackermann-Liebrich et al. 1997). Likewise, a study on air pollution on lung function, inflammation and aging (SALIA) showed a fall in FEV_1 of 4.7 %, in FVC of 3.4 %, and in FEV_1/FVC of 1.1 % during the same increase in air pollutants (Schikowski et al. 2005). Prospective cohort studies show that a decrease, on average, in spirometric indices is less significant in persons living in less polluted areas compared with those exposed to high concentrations of sulfates, nitrogen oxides, and hydrocarbons (Tashkin et al. 1994).

The objective of the present study was to examine the relative risk of lung obstruction in people older than 40 years living close to urban roads with intense traffic. To this end, we compared pulmonary function in a group of persons exposed long-term to statistically greater concentrations of air pollutants, such as CO, NO_2, or PM_{10} ($p < 0.05$), with that in a control group consisting of persons living in rural areas.

2 Methods

The study was approved by an institutional Research Review Board. It consists of two parts: the measurements of air traffic-related

pollutant concentration and of pulmonary function. The measurements were done in the city of Warsaw, Poland, in seven predefined locations in 2008–2012. The road sections, selected for the study were typical urban canyons characterized by severe traffic intensity and congestion. A limited dispersion of air pollution, compared with other road fragments, resulted in a similar level of air pollution in the total section investigated. Nevertheless, there is a building storey-dependent difference in the concentration of air pollutants due to a vertical gas and particulate dispersion in urban canyons.

The air pollution measurements were taken in series, choosing months in which the dominant contributing factor was heavy road traffic (June and October) and which are not considered as a holiday or heating season. An Airpointer^{TM} mobile monitoring station for the measurement of air pollution concentration was used (MLU, Wiener Neudorf, Austria). The measurements were conducted for 24 h, 7 days a week. The measuring equipment was localized about 1 m from the edge of the street. The device registered the following concentrations: carbon monoxide, nitrous oxides, volatile organic compounds, and the PM_{10} suspended particle. The concentration of PM_{10} was measured using a nephelometric method, which is based on the measurement of light dispersion in relation to the concentration of solid particles with a diameter of >10 μm. The urban sections selected were characterized by high traffic congestion; from 30,000 to 70,000 vehicles per day. In addition to seven sections in Warsaw, measurements also were done in two control points localized in the cleanest, from the standpoint of air pollution, areas of Eastern Poland. The control points were at least 250 m away from roads, and the traffic congestion was of one order of magnitude lower than that in Warsaw.

Respiratory function was studied from April to June and from September to October. The choice of the study period excluded the winter heating periods that cause a passing increase in air pollution, which could affect the functional results. The other reason was to avoid a holiday season, during which the sample would not be representative. Pulmonary function was assessed with an EasyOne spirometer (ndd Medizintechnik AG, Zürich, Switzerland). This device is compatible with the international standards of the European Respiratory Society (ERS). The following parameters were measured: functional vital capacity (FVC), forced expired volume in 1 s (FEV_1), FEV_1/FVC, peak expired flow (PEF), and forced expiratory flow at 50 % (FEF_{50}). The measurement was followed by a questionnaire that provided information on age, place, and duration of residence, tobacco smoking habits, and respiratory symptoms. The period of residence at a location was stratified into 20, 30, and 40 years to assess the variability in the occurrence of lung obstruction caused by prolonged environmental exposure to pollutants.

Smoking persons and those aged below 40 were excluded from further study procedures. The effect on the incidence of lung obstruction of increased PM_{10} alone could be estimated. The exclusion of younger than 40 years persons was due to the above mentioned prolonged exposure paradigm used. The GOLD guidelines (2011) for the interpretation of pulmonary function were used to determine the incidence of obstructive disease. Lung obstruction was diagnosed when the FEV_1/FVC ratio was less than 0.7. Post-bronchodilator tests or reversibility of bronchoconstriction were not assessed. Therefore, no distinction between asthma and COPD could be done and the results pertain to general lung obstruction.

The results of measurements of PM_{10} concentrations were used to determine average exposure of people to particulate matter, both in urban conditions and the control area. The study was not conducted throughout the entire year; thus, the average annual PM_{10} concentration could not be calculated. Nevertheless, substantial differences in the concentration of pollutants between testing sites was observed. Taking that into account, a general exposure level of PM_{10} for certain groups of habitants could be determined, although an accurate estimation of a difference between the study results and the actual mean annual concentration was not possible.

Despite this limitation, it is important to underscore that the measurement of air pollution in close proximity to roads seems more accurate than the actual data provided by the National Environmental Monitoring, which has monitoring stations in distant locations from the sites chosen for this study. Furthermore, the accuracy of the present data was verified by the determination of confidence intervals for exposure-response functions.

Taking into account the GOLD guidelines (2011) and the three defined periods of residence, it was possible to determine the relative risk (RR) of the incidence of lung obstruction in the groups studied. As a result, three patterns of exposure-response functions were obtained. The first step in RR calculation was to determine the incidence number within each group, and to estimate the probability of the occurrence of lung obstruction. By relating these results to the control group, it was possible to define particular RR for each of the seven measurement sites. Because different groups were exposed to different PM_{10} concentrations, the results were normalized for inter-group comparisons by matching the RR of lung obstruction with the concentration increase of PM_{10} by 10 μg/m^3. The final step was to determine the confidence intervals and mean concentration results.

3 Results

Daily fluctuations in PM_{10} concentration were observed in the city road traffic sections studied, particularly present when PM_{10} increased during rush hours. Nevertheless, the mean concentrations of PM_{10} in all seven stations was an order of magnitude greater than its level at the two control points; highly distinct differences ($p < 0.05$) (Table 1). Pearson's linear correlation between PM_{10} concentration and traffic congestion was calculated. The correlation coefficients were significant ($p < 0.05$), varying from 0.53 to 0.72, showing that traffic congestion had a strong impact on the increase in PM_{10}.

A total of 4,985 persons (3,997 living in urban areas and 988 control subjects from non-urban areas) were subjected to respiratory function tests followed by questionnaires. A number of persons had to be discarded from final analysis due to insufficient technical quality of test results (short forced expiration, improper placement of the mouthpiece, and improper position of the tongue during the test), unambiguous interpretation of results, or lack of proper cooperation of the persons studied. Tobacco smokers were excluded as well, to avoid a possible bias in determining the influence of air pollution on respiratory function. Additionally, persons aged below 40 were also excluded from further analysis, since the incidence of lung obstruction in this age category is low. After the exclusions, there remained 2,378 persons living in urban areas in the vicinity to busy traffic roads and 762 control persons from outside such areas, whose results qualified for further analysis. These study persons were stratified into three groups depending on the period of residence at either location: 20, 30, and 40 years. The respective groups encompassed 1,031, 797, and 550 persons from polluted areas and 296, 249, finally 208 persons from control areas. Detailed results in regard to each of the seven city road traffic sections and two control points (C) are presented in Table 2.

A number of residents at a given location were decreasing with the extension of the period of living at a given location, which may have affected the accuracy of the estimation of the relative risk of lung obstruction. Also, the control group was, on average, junior to persons living in the areas of heavy exposure to pollution. In general, the incidence of lung obstruction decreased as the period of residence increased, with the exception of the control group. However, as the population number decreased with longer living at a location as above mentioned, the probability

Table 1 Mean PM_{10} concentrations measured at seven city road traffic sections studied and at two control points (C)

Road traffic stations	1	2	3	4	5	6	7	C1	C2
PM_{10} concentration (μg/m^3)	41.1	39.3	32.5	31.0	58.7	49.3	48.1	7.9	6.2

Table 2 Incidence of lung obstruction at seven city road traffic sections studied and at two control points (C) stratified by the period of residence at a given location

Road traffic stations	1	2	3	4	5	6	7	C1	C2
20 years' residence									
n	140	181	108	124	151	167	160	206	90
Mean age ± SD (year)	71.0 ± 11.5	67.5 ± 13.1	60.3 ± 15.1	68.0 ± 13.4	67.5 ± 12.0	68.1 ± 11.6	66.6 ± 11.5	61.1 ± 13.9	59.4 ± 13.3
Obstruction incidence	64	71	36	36	47	54	53	41	15
30 years' residence									
n	111	141	59	92	127	142	125	175	74
Mean age ± SD (year)	72.8 ± 10.0	70.6 ± 9.9	67.9 ± 10.7	71.9 ± 9.1	69.4 ± 10.7	70.6 ± 9.1	69.2 ± 9.9	64.7 ± 11.7	63.4 ± 10.8
Obstruction incidence	52	60	25	29	42	46	45	41	12
40 years' residence									
n	77	95	44	68	90	95	81	156	52
Mean age ± SD (year)	75.5 ± 9.1	70.9 ± 10.7	71.1 ± 10.0	72.3 ± 8.8	71.3 ± 10.5	72.1 ± 9.0	70.0 ± 9.8	64.9 ± 11.8	65.5 ± 11.1
Obstruction incidence	39	43	18	22	31	29	33	41	9

of lung obstruction actually increased in both studied and control groups. The study also suggests that in the control groups, not exposed to high concentrations of air pollution, the risk of lung obstruction is less related to the residence period, but the influence of person's age assumes a bearing. It means that the probability of lung obstruction in these groups depends on the correlation between the person's increasing age and the length of residence to a greater degree than in the other groups studied.

Finally, we determined the relative risk (RR) of lung obstruction in response to increased PM_{10} concentration. The probability of the occurrence of lung obstruction in each group was determined and related to the control group by the following formula:

$$RR_i = \frac{p_i}{p_0}$$

Where:

RR_i is the relative risk of lung obstruction in i group at i concentration of PM_{10};

p_i is the probability of lung obstruction in i group at i concentration of PM_{10};

p_0 is the probability of lung obstruction in the control group.

Since each group studied was exposed to a different PM_{10} concentration, the results were recalculated to normalize the relative risk of lung obstruction to an increase in PM_{10} by 10 μg/m^3. The risk of lung obstruction for each residence period is presented in Table 3 and the statistical elaboration of these data is displayed in Fig. 1.

The results of the relative risk of lung obstruction in response to an increase in PM_{10} by each 10 μg/m^3 demonstrate a wide spread of values around the median, where the maximum risk is 1.45 and the minimum is 1.00, and rather an opposite to the expected trend for reduced risk as the residence period increases. There is a small difference in the number of people with lung obstruction in the control groups. However, when the number of people decreases, the probability of lung obstruction increases. In case of the urban groups, the number of people with lung obstruction decreases, as the length of residence increases. That means that people exposed to high concentrations of PM_{10} become affected by lung obstruction at a much lower age than those living in the areas of low PM_{10} concentration. That spurious decrease in the incidence of lung obstruction in urban citizens, along with little changes in the control groups, caused an overall decrease in the relative risk. Nonetheless, the results demonstrate a positive relative risk of lung obstruction due to an exposure to PM_{10}. The substantial spread of the relative risk underscores that different diagnostic recommendations might affect the final results.

Table 3 Relative risk of lung obstruction after an increase in PM_{10} by 10 μg/m^3

Residence period in the vicinity to road traffic sections studied (year)	Relative risk of lung obstruction normalized to an increase in PM_{10} by 10 μg/m^3
20	1.27
30	1.24
40	1.19

Lung obstruction was defined according to GOLD guidelines (GOLD 2011)

4 Discussion

A long-term exposure to air pollution caused by high concentrations of PM_{10} in close proximity to roads characterized by high traffic volume and traffic congestion may increase the risk of COPD. It also has a negative effect on lung function (Schikowski et al. 2005). The present study demonstrates that there is a relationship between a long-term concentration of air pollutants, with the PM_{10} level taken as a surrogate, and the incidence of lung obstruction, which is demonstrated by the calculated values of the relative risk factor.

In the presented study, as opposed to other studies, the relative risk was related to lung obstruction, instead of mortality. That seems of importance in view of the ambiguity of results from studies analyzing the interconnection between risk and mortality from respiratory and other diseases related to particles suspended in the air (Pope et al. 2004). The problem may lie in the complex and not fully understood

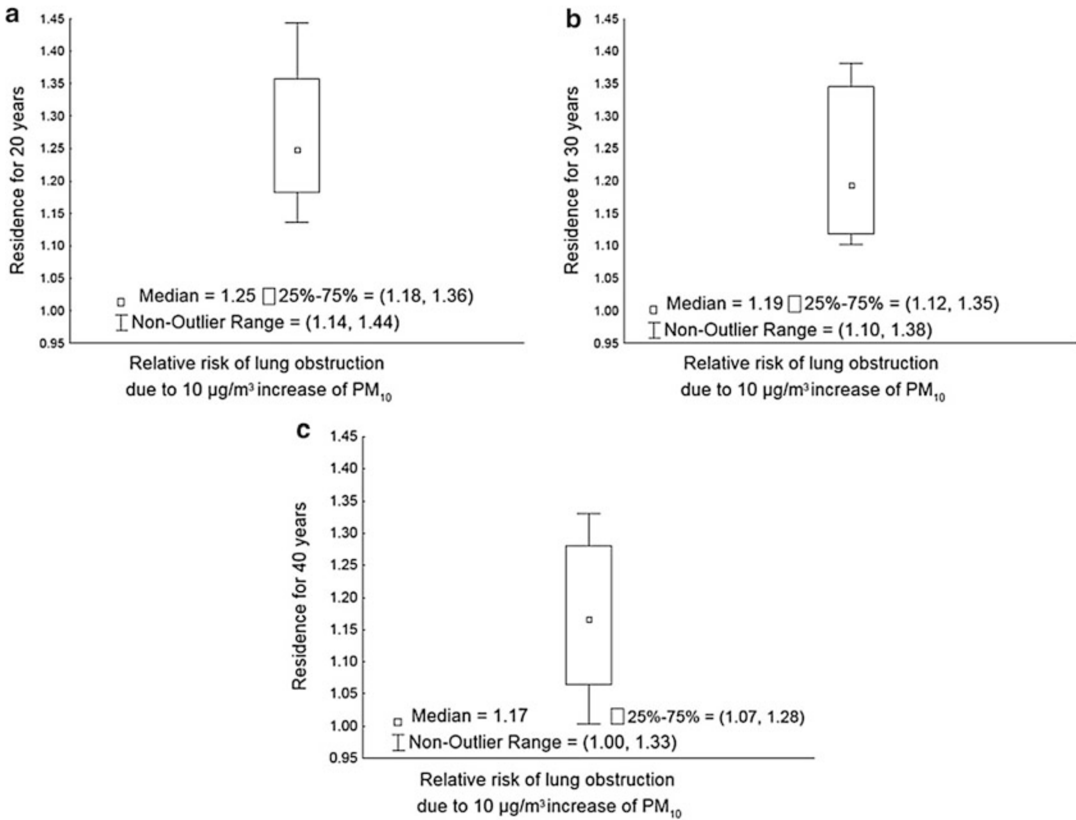

Fig. 1 Median and spread of the relative risk of lung obstruction in response to an increase in PM_{10} by 10 μg/m^3

pathophysiological pathways connecting the exposure to suspended particles to mortality from cardiopulmonary disorders. The present study also is based on actual data tallied in a big bustling city with a high emission of PM_{10} and contrasting rural areas, rather than on purely statistical data.

Generally, the relative risk varies depending on the characteristics of groups studied, the type of contamination, and the time of exposure. There is, however, one common feature of different analyses of the matter. The results always confirm, although to a different degree, that air pollution, especially one caused by suspended particles, have a significant impact on the increase of lung obstruction and related mortality. A case in point is the Schikowski et al. (2005) study, which is similar in assumptions to the present article, but describes a specific type of lung obstructive disease – COPD. That study shows that a decrease in respiratory capacity and an increase in COPD incidence are caused by exposure to PM_{10} and other pollutants characteristic of road traffic. A decrease in FEV_1 by 5.1 % (95 % CI 2.5–7.7) has been related to an increase in the mean PM_{10} (interquartile range) by 7 μg/m^3 within 5 years. A corresponding decrease in FVC was 3.7 % (95 % CI 1.8–5.5) and the odds ratio for COPD was 1.33 (95 % CI 1.03–1.72). In a different study, concerning the short-term exposure to pollution stemming from suspended particles, it has been demonstrated that an increase in the 24-h concentration of $PM_{2.5}$ by 10 μg/m^3 increases the relative risk of daily mortality from heart disease by 0.4 to 1.0 % (Brook et al. 2010). That study revealed that it is worthwhile to look at the absolute risk, which may be a more effective way of presenting the information about health hazards, particularly that statistically calculated relative risk is not

evenly spread within the population. The relative risk is, however, greater for persons who live in areas of higher concentrations of $PM_{2.5}$ for long time than for those affected in the short-term; 1.76 and 1.06, respectively, for a change of 10 μg/m^3 (Dockery 2006; Brook et al. 2010). Short-term increases in $PM_{2.5}$ should not be neglected, because they might lead to tens of thousands of deaths in the US alone. Nonetheless, studies on the adverse health effects of daily exposure to increased concentrations of suspended particles do not seem to provide sufficient information on the reduction in life expectancy, the influence of air pollution on long-term mortality, or the role of pollutants in the occurrence and progression of chronic diseases (McMichael et al. 1998).

5 Conclusions

The present study confirms the impact of increased PM_{10} concentration by each 10 μg/m^3 on the risk of lung obstruction. The study covered a population sample older than 40 years, in which the risk of obstructive lung diseases is higher than that of asthma. We took into account only non-smoking persons, unaffected by this key factor of lung obstruction. The calculated relative risk of lung obstruction amounted to 1.27, 1.24, and 1.19 for persons living in urban areas exposed to an average increase in PM_{10} emission of 10 μg/m^3 for 20, 30, and 40 years, respectively. A tendency for a decrease in the risk of obstruction was revealed, opposing the expectancy, which seems spurious as it indicates that in fact lung obstruction develops faster in the groups living shorter in the polluted air. That spurious decrease in the incidence of lung obstruction in urban citizens, along with little changes in the control groups, caused an overall decrease in the relative risk. The study shows a positive relative risk of lung obstruction due to an exposure to high PM_{10} emission.

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

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