Physical activity in asthma control and its immune modulatory effect in asthmatic preschoolers

Debbie Maurer[1], Chengyao Liu[1], PARASKEVI XEPAPADAKI[2], Claus Bachert[3], Susetta Finotto[4], Ya-dong Gao[5], Anna Graser[4], Tuomas Jartti[6], Walter Kistler[7], Marek Kowalski[8], Heikki Lukkarinen[6], Maria Passioti[9], Barbara Stanic[10], Ge Tan[11], Michael Villiger[7], Luo Zhang[12], Nan Zhang[13], Mübeccel Akdis[14], Nikolaos Papadopoulos[15], and Cezmi Akdis[14]

[1]University of Zurich Swiss Institute of Allergy & Asthma Research
[2]National and Kapodistrian University of Athens
[3]Upper Airway Research Laboratory (URL),
[4]Friedrich-Alexander-Universitat Erlangen-Nurnberg
[5]Zhongnan Hospital of Wuhan University
[6]Turku University Hospital
[7]Hospital Davos
[8]University of Łódź
[9]University of Athens
[10]University of Zürich
[11]Swiss Institute of Allergy and Asthma Research
[12]Beijing TongRen Hospital, Capital Medical University,
[13]Upper Airway Research Laboratory (URL)
[14]University of Zurich
[15]Second Pediatric Clinic

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Abstract

Background: The impact of physical activity (PA) on immune response is a hot topic in exercise immunology, but studies involving asthmatic children are scarce. We examine the level of PA and TV attendance (TVA) in asthmatic children to assess the role on asthma control and immune response to various stimulants. Methods: Weekly PA and daily TVA were obtained from questionnaires at inclusion of the PreDicta study. PBMC cultures were stimulated with phytohemagglutinin (PHA), R848, poly I:C and zymosan. Cytokines were measured and quantified in cell culture supernatants using luminometric multiplex immunofluorescence beads-based assay. Results: Asthmatic preschoolers showed significantly more TVA than their healthy peers (58.6% vs. 41.5% 1-3h daily and only 25.7% vs. 47.2% [?] 1h daily). Poor asthma control was associated with less frequent PA (75% no or occasional activity in uncontrolled vs. 20% in controlled asthma; 25% [?] 3x weekly vs. 62%). Asthmatics with increased PA exhibited elevated cytokine levels in response to stimulants, suggesting a readiness of circulating immune cells for type-1, -2 and -17 cytokine release compared to low-PA and high-TVA subjects. Low PA and high TVA were associated with increased proinflammatory cytokines. Proinflammatory cytokines were correlating with each other in in-vitro immune responses of asthmatic children, but not healthy controls. Conclusion: Asthmatic children show more sedentary behavior than healthy subjects, while poor asthma control leads to a decrease in PA. Asthmatic children profit from exercise, as elevated cytokine levels in stimulated conditions indicate an immune system prepared for a strong response in case of infection.

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Short title: Physical activity, immune response and pediatric asthma

Authors: Debbie J. Maurer[1,2,3], Chengyao Liu[1,4], Paraskevi Xepapadaki[5], Claus Bachert[6], Susetta Finotto[7], Ya-dong Gao[8], Anna Graser[7], Tuomas Jartti[9,10,11], Walter Kistler[2,3], Marek Kowalski[12], Heikki Lukkarinen[9], Maria Pasioti[5], Barbara Stanic[1], Ge Tan[1], Michael Villiger[2,3], Luo Zhang[4,13], Nan Zhang[6], Mübeccel Akdis[1], Nikolaos G. Papadopoulos[5,14], Cezmi A. Akdis[1,2,15]

[1] Swiss Institute of Allergy and Asthma Research (SIAF), University of Zurich, Davos,

Switzerland

[2] Swiss Research Institute for Sports Medicine (SRISM), Davos, Switzerland

[3] Department of Sports Medicine, Davos Hospital, Davos, Switzerland

[4] Department of Otolaryngology Head and Neck Surgery, Beijing Tongren Hospital, Capital Medical University, Beijing, China

[5] Allergy Department, 2nd Pediatric Clinic, National and Kapodistrian University of Athens, Athens, Greece

[6] Upper Airway Research Laboratory, Ghent University Hospital, Ghent, Belgium

[7] Department of Molecular Pneumology, Friedrich-Alexander-Universität (FAU) Erlangen-Nürnberg, Universitätsklinikum Erlangen, Erlangen, Germany

[8] Department of Allergology, Zhongnan Hospital of Wuhan University, Wuhan, China

[9] Department of Pediatrics and Adolescent Medicine, University of Turku and Turku University Hospital, Turku, Finland

[10] PEDEGO Research Unit, Medical Research Center, University of Oulu, Oulu, Finland

[11] Department of Pediatrics and Adolescent Medicine, Oulu University Hospital, Oulu, Finland

[12] Department of Immunology, Rheumatology and Allergy, Central University Hospital, Lodz, Poland

[13] Department of Allergy, Beijing Tongren Hospital, Capital Medical University, Beijing, China

[14] Division of Infection, Immunity & Respiratory Medicine, University of Manchester, Manchester, UK

[15] Christine Kühne-Center for Allergy Research and Education, Davos, Switzerland

Maurer DJ and Liu C equal first-author contribution.

Akdis M, Papadopoulos NG and Akdis CA are shared last authors.

Corresponding author:

Cezmi A. Akdis

Swiss Institute of Allergy and Asthma Research (SIAF), University of Zurich, Davos,

Switzerland, Herman-Burchard-Strasse 9, 7265 Davos, Wolfgang, Switzerland

akdisac@siaf.uzh.ch, Tel +41 81 4100848

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Abstract

Background : The impact of physical activity on immune response is a hot topic in exercise immunology, but studies involving asthmatic children are scarce. Our aims were to examine whether there were any

differences in the level of physical activity and daily TV attendance, to assess its role on asthma control and immune responses to various immune stimulants.

Methods: Weekly physical activity and daily television attendance were obtained from questionnaires at inclusion of the PreDicta study. PBMC cultures were stimulated with phytohemagglutinin (PHA), R848, poly I:C and zymosan. A panel of cytokines was measured and quantified in cell culture supernatants using luminometric multiplex immunofluorescence beads-based assay.

Results: Asthmatic preschoolers showed significantly more TV attendance than their healthy peers (58.6% vs. 41.5% 1-3h daily and only 25.7% vs. 47.2% [?] 1h daily) and poor asthma control was associated with less frequent physical activity (PA) (75% no or occasional activity in uncontrolled vs. 20% in controlled asthma; 25% [?] 3 times weekly vs. 62%). Asthmatics with increased PA exhibited elevated cytokine levels in response to polyclonal stimulants, suggesting a fitness and readiness of circulating immune cells for type 1, 2 and 17 cytokine release compared to subjects with low PA and high TV attendance. Low physical activity and high TV attendance were associated with an increase in proinflammatory cytokines. Proinflammatory cytokines were correlating with each other in *in vitro* immune responses of asthmatic children, but not healthy controls.

Conclusion: Asthmatic children show more sedentary behavior than healthy subjects, while poor asthma control leads to a substantial decrease in physical activity. Our results suggest that asthmatic children profit from regular exercise, as elevated cytokine levels in stimulated conditions indicate an immune system prepared for responding strongly in case of different types of infections.

Keywords: asthma; cytokines; immune modulation; physical activity; PreDicta

Introduction

Asthma affects more than 300 million people worldwide, and it is estimated that this number will increase to over one billion by 2050.[1] It is more prevalent in children, where it lies among the top 20 chronic diseases for the global ranking of disability-adjusted life years[1,2] with up to 25% of children affected in Western urban areas.[3] The global burden of asthma in children and adolescents increased significantly over the last decades, while previously countries affected at lesser extent in Africa, South America and Asia started to catch up on these numbers.[1,4] Multiple factors are thought to play a role in this world-wide increase in asthma prevalence including allergen exposure and sensitization, environmental influences, viral infections, urbanization, diet and sedentary lifestyle with physical inactivity.[5,6] Asthma attacks often appear during physical activity (PA),[7] termed "exercise-induced asthma" (EIA) affecting 70-90% of asthmatic children.[3]

PA is beneficial both for growth and the psychological development of children.[8-10] Whereas the World Health Organization (WHO) recommends for children and adolescents aged 5 to 17 years to be moderately to vigorously active for a minimum of 60 minutes daily,[10,11] specific guidelines for children suffering from asthma are lacking.[12] Although it is agreed that it is essential for asthmatic children to participate in sports,[7] fear of EIA might prevent the practice of regular PA in children, particularly the ones with severe and/or uncontrolled asthma.[3,13] This self-limiting cycle of inactivity has led to the common perception that asthmatics are more physically inactive in comparison to healthy individuals.[3]

Asthma pathogenesis is a consequence of epithelial barrier dysfunction,[14] and research has shown that there are several underlying pathophysiological mechanisms (endotypes) that can lead to variable clinical presentations (phenotypes).[15-17] An imbalance of both Th1/Th2 cells and Th17/Treg cells can play a crucial role in its development.[18-20] Type 2 inflammation is considered to be the major driver in the most common phenotype, allergic asthma,[21] and seems to be the result of a complex cross-talk between airway epithelium, innate and adaptive immunity.[19] PA is known to impact both the innate and adaptive immune responses in various ways: For example, moderate exercise was found to lead to an acute increase in the absolute numbers of natural killer (NK) cells and NK cell cytotoxicity.[22,23] As for the adaptive immune response, increased and intensified training loads were shown to lead to a reduction of T cell functionality in well-trained individuals, with lower numbers of circulating type 1 T cells, reduced T cell proliferation responses

and B cell immunoglobulin synthesis.[22] While Walsh et al. describe in their position statement on immune function and exercise a resulting temporary inhibition of Th1 cytokine production in trained individuals without asthma, studies examining the impact of aerobic exercise in murine asthma models reported an enhancement of Th-1 and Treg responses and lower levels of Th-2 cytokines.[22,24,25]

The "Post-infectious immune reprogramming and its association with persistence and chronicity of respiratory allergic diseases" (PreDicta) study, was designed to prospectively evaluate asthma persistence in preschoolers in association with microbial exposures and immunological responses.[26] PreDicta has demonstrated differential immune responses to viruses in asthma,[27,28] as well as evolution of airway inflammation at that age.[29] The objective of the present study was i) to evaluate whether there were any differences in the level of PA and sedentary behavior between asthmatic and non-asthmatic children, ii) to assess the impact of asthma control on PA and iii) to examine the influence of PA and long time TV attendance on the immune system in asthmatic children. We have investigated multiple cytokine levels in response to four different immune stimuli: by phytohemagglutinin (PHA) that mimics polyclonal innate immune activation, by polyinosinic-polycytidylic acid (poly I:C) and R848 (resiquimod) that both mimic respiratory virus stimulation and by zymosan (zymo) that mimics fungal stimulation in PBMC cultures. R848 is an imidazoquinoline compound with potent antiviral activity that functions through the Toll-like receptor (TLR)7/TLR8 myeloid differentiation primary response protein (MyD88)-dependent signaling pathway.[30] Poly I:C is a synthetic analog of double-stranded RNA that induces the production of pro-inflammatory cytokines by the activation of NF-κB through TLR3.[31]Zymosan is a glucan binding to TLR2 and dectin1 found on the surface of yeasts.[32]

Methods

2.1 Study population & design

Our analyses were carried out within the framework of the PreDicta study, a 2-year multi-center prospective cohort study that has been conducted across five major European cultural and climatic regions (Greece, Germany, Belgium, Poland and Finland).[26]

We included 140 pre-school children aged 4-6 years with a diagnosis of mild to moderate asthma confirmed by a doctor (in the preceding 2 years, according to the GINA guidelines 2005) as cases, which were previously recruited for the PreDicta study. Eligibility criteria for the PreDicta study included the ability to perform a peak expiratory flow (PEF) measurement at least once and a minimum of 3 wheezing episodes within the last 12 months prior to study inclusion (one of them within the last six months). Exclusion criteria were severe asthma, more than six courses of oral steroids during the last 12 months prior to study inclusion, immunotherapy, chronic medication use or the history of chronic respiratory disease other than asthma and/or allergic rhinitis (e.g. cystic fibrosis). Additional exclusion criteria were an asthma exacerbation and/or upper respiratory tract infection within 4 weeks prior to study inclusion. The cross-sectional control group consisted of 53 healthy, age-matched children with no history of asthma/wheezing or any other allergic disease.

At recruitment, a detailed questionnaire on demographic characteristics, as well as asthma activity and control was handed out. Asthma was classified as uncontrolled, partly controlled or controlled according to the GINA guidelines[12]. As part of the questionnaires, weekly vigorous PA and daily television attendance (TVA) were assessed. PA with an intensity higher than 4 metabolic equivalents (METs) was classified as vigorous and included brisk walking, bicycling, gymnastics, dancing, basketball, soccer, athletics, tennis, swimming, jumping rope and general participation in active outdoors games. Such activity had to be continuous and cause sweating and heavy breathing for periods longer than 15 minutes.

Whole blood was obtained at baseline for the analysis of cytokine responses of peripheral blood mononuclear cells (PBMCs) cultured in the presence of various stimuli.

Written informed consent was obtained from parents/guardians of all the children recruited in the PreDicta study. The study protocol was approved by the local Ethics Committee of all participating institutions.

2.2 Blinding of study subjects

The study participants were assigned individual study numbers, in which the first digit stands for the respective study center (1: Athens, 2: Erlangen, 3: Ghent, 4: Lodz, 5: Turku). The participants' full name could only be accessed by the clinical investigator and study nurses of the respective Childrens' Hospital.

2.3 *In vitro* culture of peripheral blood mononuclear cells

Blood samples were collected in tubes with lithium heparin (Vacutainer®) and diluted with an equal volume of warm PBS (Gibco, Invitrogen, Massachusetts). PBMCs were isolated by centrifuging at 800g for 20 min at 18-20°C on Biocoll separating solution (Biochrom AG, Germany). PBMCs were washed three times and the cell pellet was resuspended in complete medium [RPMI 1640 with HEPES 25 mM and L-Glutamine (Gibco, Life Technologies Ltd, UK), supplemented with 10ml/L Penicillin-Streptomycin USA, 50µl/L 1M β-mercaptoethanol, 20ml/L L-Glutamine plus MEM Vitamin, 20ml/L Non-essential Amino Acid, Sodium Pyruvate and 10% heat-inactivated FBS (all from Sigma-Aldrich, Germany)]. The suspension was seeded in a flat-bottom 48-well tissue plate (Corning Incorporated, Costar, New York), with 5×10^5 viable cells per well (500µL). PBMCs were cultured in duplicates either with complete medium alone (unstimulated control) or with one of the following stimulants: 4µg/ml Resiquimod (R848), 10µg/ml Zymosan (InvivoGen, France), 10µg/ml Phytohemagglutinin (PHA-M), 20µg/ml Polyinosinic–polycytidylic acid potassium salt (Poly I:C), (Sigma-Aldrich, Germany), at 37°C, 5% CO_2. Cultures were harvested after 48 hours and, after centrifugation at 600g for 5 min, supernatants were stored at -80°C until analysis.

2.4 Detection of cytokines secreted in PBMCs culture supernatants

Cytokine expression levels in the supernatants of stimulated cultures were quantified by multiplex bead-based fluorometric immunoassay (Milliplex, Millipore) using Luminex xMAP technology (Luminex 200, Bio-Rad) at the Swiss Institute for Allergy and Asthma Research (SIAF) in Davos, Switzerland. Values out of range were either marked as the lowest (those on a lower end) or the highest (those on a higher end) detectable values. The panel used contained IFNα2, IFNγ, IFNλ-2, IL-1β, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-23A, IL-25, IL-27, IL-33, CCL3, CCL4, CCL5, CXCL8, CXCL10, TNF-α. The T-cell subset classification of all measured cytokines can be found in Online Supplementary Table 1.

2.5 Statistical analysis

Nominal variables are presented as absolute and relative (%) frequencies. Continuous variables are presented in violin plots to show frequency distribution, with median and quartiles. Data was evaluated for significant statistical differences using i) the Chi-square test to evaluate associations between two nominal variables, ii) the non-parametric Kruskal-Wallis test for multiple comparisons and iii) the non-parametric Mann-Whitney test for independent sample tests (Prism 8.0 for macOS; GraphPad Software, San Diego, CA, USA). Correlation was assessed with the Spearman method. A local regression fitting line was added to the scatter plot with a confidence level of 95%. Statistical significance was set at $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

Results

3.1 Baseline characteristics

From 167 cases and 66 controls that were recruited for the PreDicta cohort, a total of 140 asthmatic children (82 males, 58%) and 53 healthy controls (22 males, 42%) were included in this study. The reason for the exclusion of 27 subjects was insufficient blood samples for cell culture experiments. The respective number of cases and controls per study center is: Athens 36/13, Erlangen 22/15, Ghent 20/13, Lodz 25/4 and Turku 37/8. Mean age at recruitment was 5.3 ± 0.7 in asthmatic and 5.0 ± 0.8 in healthy children. The majority of asthma cases additionally suffered from allergic rhinitis (63.5%) and more than half of them (52.1%) reported atopic dermatitis, while 31.4% had experienced adverse food reactions.

Table 1 shows demographic characteristics, weekly vigorous PA and daily TVA as well as asthma control and allergic comorbidities, as gathered through questionnaires at inclusion.

3.2 Physical activity and TV attendance in asthmatic preschoolers and the effect of asthma control

In the asthmatic group, a significantly higher daily TVA was reported (daily TVA <1h in 25.7% of cases versus 47.2% of controls; 1-3h daily TVA in 58.6% of cases and 41.5% of controls, Table 1). Also, there was a higher percentage of children that were never or only occasionally vigorously physically active in the case group (30.0% versus 24.5% of healthy subjects), whereas the control group showed more PA ([?] 3 times of vigorous PA per week in 56.6% of controls versus 52.8% of cases). However, the differences in weekly PA were not statistically significant.

Children with poor asthma control were significantly more physically inactive (75.0% never or occasionally physically active versus 25% [?] 3 times per week, Table 2 and Fig. 1A). In contrast, there was a significantly higher proportion of physically active subjects in those with good asthma control (62% of them with PA [?] 3 times per week and 18% 1-2 times per week).

Daily TVA was slightly higher in children with uncontrolled asthma showing 81% > 1 h TVA per day in the uncontrolled asthma group compared to 78% and 70% in the partly controlled asthma and controlled asthma groups, respectively (Fig. 1B). However, no statistical significance was shown for this observation.

3.3 Cytokine levels under different stimulations in asthmatic preschoolers

No significant differences could be observed for baseline cytokine levels between healthy and asthmatic subjects, as displayed in Online Supplementary figure S-Fig. 1.

Asthmatic children showed high numbers of cytokines increased in response to various stimuli compared to healthy subjects (Fig. 2A). Particularly, R848 and poly I:C were the stimulants that induced IL-10, IL-13, IL-25, IFNγ, CCL4 and CXCL10, while stimulation with PHA and zymo induced IFNα2 and CCL4, respectively.

In contrast, only IL-7 and IFNλ-2 were found to be lower in asthmatic in comparison to healthy subjects after stimulation with PHA and poly I:C (Fig. 2B). Cytokines that showed no significant difference between the two groups after polyclonal stimulation are found in Online Supplementary (S-Fig. 2 - S-Fig. 5).

3.4 Impact of physical activity and TV attendance on unstimulated cytokine levels

In unstimulated conditions, levels of IL-5, IL-7, IL-9, IL-17A, IL-23A, CCL4 and CXCL10 were significantly higher, and levels of IL-1β, IL-6 and IL-25 significantly lower in asthmatic children who were more physically active compared to those who never or only occasionally exercised (Fig. 3A). In healthy subjects, increased cytokine levels with higher weekly PA could only be observed for IL-17A, TNF-α, CCL3 and CCL4 (Fig. 3C).

Again, in unstimulated conditions, high daily TVA decreased levels of IL-12p70, IL-25, IL-33 and IFNλ-2 in asthmatic children (Fig. 3B). A more sedentary behavior increased IL-1β, IL-6, IL-7, IL-17A, IL-23A, IL-27, CCL3 and CCL4 in healthy children, whereas IL-33 levels decreased (Fig 3D). Cytokines that showed no significant difference in baseline levels between the two groups according to PA and TVA are found in Online Supplementary (S-Fig. 6 - S-Fig. 7).

3.5 Impact of physical activity on cytokine levels of polyclonally stimulated PBMCs

Asthmatic children with high PA showed elevated levels of IL-1β, IL-5, IL-6, IL-7, IL-9, IL-12p70, IL-13, IL-17A, IL23A, IL-27, TNF-α, CCL4, CCL5, CXCL8 and CXCL10 in response to stimulation with PHA, poly I:C, R848 and zymo (Fig. 4A). A significant decrease was only observed for IL-10 in PBMCs stimulated with R848 and for IL-25 after stimulation with PHA, poly I:C and zymo.

In healthy children, high PA increased the levels of IL-5, IL-7, IL-10, IL-17A, IL-33, TNF-α, CCL3, CCL4 and CXCL10 after PBMC stimulation with PHA, poly I:C, R848 and zymo (Fig. 4B). Cytokines that showed no significant difference between asthmatic and healthy subjects according to their PA level are found in Online Supplementary (S-Fig. 8 - S-Fig. 11).

3.6 Impact of TV attendance on stimulated cytokine levels of polyclonally stimulated PBMCs

Stimulation with PHA and R848 led to higher levels of IL-10 in asthmatics with high daily TVA (Fig. 5A). Elevated levels were also observed for CXCL8 after stimulation with zymo. A decrease in secretion of IL-1β, IL-6, IL-12p70, IL-13, IL-17A, IL-25, IL-33, IFNα2, IFNγ and CXCL10 was observed in response to stimulation with PHA, poly I:C, R848 and zymo.

Daily long-time TVA in healthy children increased levels of IL-7, IL-12p70, IL-23A, TNF-α, IFNα2, IFNγ, CCL3, CCL5 and CXCL10 in PBMC cultures stimulated by PHA, poly I:C, R848 and zymo. A slight reduction in IL-33 and CXCL8 levels was observed for healthy subjects with longer TVA after stimulation with zymo and PHA, respectively. Cytokines that showed no significant difference between asthmatic and healthy subjects according to their daily TVA are found in Online Supplementary (S-Fig. 12 - S-Fig. 15).

3.7 Correlation of unstimulated and stimulated cytokines in preschoolers with low physical activity and high TV attendance

Asthmatic children with no or only occasional PA and / or TVA [?] 3 hours per day showed high correlations of proinflammatory cytokines (Fig. 6A and 6C), whereas barely any clusters of correlation could be identified in healthy children of the same PA and TVA groups (Fig. 6B and 6D).

Cytokines that showed particularly high correlations in asthmatics include IL-1β, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-23A, IL-27, TNF-α, IFNα2, IFNγ, CCL3, CCL4 and CXCL10. Mostly, these highly correlating cytokines were either measured in unstimulated conditions or after PBMC stimulation with PHA, poly I:C and R848.

Discussion

In the present study, we investigated the immunological effects of PA and asthma control. Children with controlled asthma engaged in vigorous PA considerably more often compared to those with *partially controlled* or *uncontrolled* asthma. In addition, asthmatic preschoolers reported more daily TV hours compared to their healthy peers. As a general finding, physically active asthmatics expressed higher levels of various cytokines in PBMC cultures under both unstimulated conditions and stimulation with different polyclonal stimulants, while long daily TVA was associated with an overall decrease in cytokine levels.

Asthmatics are commonly perceived as being more physically inactive in comparison to healthy individuals.[3] Significantly less TV hours in healthy children shown in the present study, which we interpret as less sedentary behavior, supports this notion. One explanation for this behavior can be described as a vicious circle: in fear of experiencing exercise-induced dyspnea, the parents or the child might consciously or unconsciously restrict his or her practice of PA.[33] Of course, such avoidance behavior particularly affects subjects with severe and/or uncontrolled asthma.[3,13] Our study supports the importance of PA in asthma control, showing that children with controlled asthma are engaged in PA significantly more often than their peers with uncontrolled asthma.

Although there was a significant difference between controlled and uncontrolled asthma, we did not find any significant difference regarding the weekly amount of PA between asthmatic and healthy children in the present study. The impact of asthma diagnosis on sports in childhood and adolescence is generally accepted. The AIRE study demonstrated that 30% of asthmatic children felt limited in their physical activities.[34] It was also reported that a majority of asthmatic children perceived the inability to participate in sports as the worst thing about their asthma.[35] So far, studies comparing the level of PA in asthmatic and non-asthmatic children have shown controversial results.[3] Whereas Firrincieli et al. found that asthmatic children are less physically active,[33] others suggested their level of PA does not differ from healthy children,[5,13,35] or that they are even more active.[36] Factors that could make for this inconsistency could be the asthma diagnosis criteria used in this age group, awareness of the doctors for suggesting PA in the cohort, regional cultural differences as well as the instruments used to quantify PA.

Exercise is known to enhance the health-related quality of life in asthmatics not only by improving aerobic capacity, but also by reducing dyspnea, the intensity of exercise-induced bronchoconstriction, the dose of

inhaled corticosteroids and exacerbation of their asthma.[13,37] Therefore, the American College of Sports Medicine and the American Thoracic Society endorse prescription of PA for all asthmatic subjects.[6] Guidelines focused on physical activity for pediatric asthma patients are lacking. A recommendation to exercise on a regular basis for children with controlled asthma is made by the Global Initiative for Asthma (GINA).[38] In addition, there has been substantial research on the use of exercise to treat asthma, which also proved to be safe and beneficial for pediatric asthmatic subjects.[39] Bonini et al. conducted a study with Italian Olympic athletes that may give young asthmatics every reason to support their PA. The authors reported that adequately diagnosed and treated asthmatic athletes can compete at the highest level.[7,40]

The present study also investigated *in vitro* immune response of asthmatic and healthy children by analyzing cytokines produced from PBMC that may contribute to asthmatic inflammation in allergic and nonallergic asthma.[15] Baseline cytokine levels of unstimulated PBMC did not differ between the two groups. For our investigation of immune responses in PBMC cultures, we chose four stimulants: phytohemagglutinin (PHA) acts as a mitogen that leads to a polyclonal immune activation, poly I:C and R848 both mimic respiratory viral infections and zymosan (zymo) mimics immune response to a fungal infection. Compared to healthy subjects, asthmatic preschoolers show higher cytokine levels particularly after stimulation poly I:C and R848, indicating a strong response in case of respiratory virus contraction. Regular exercise is further known to have anti-inflammatory effects, which most likely play an important role in its ability to reduce the risk of chronic metabolic and cardiorespiratory diseases.[23,41] The three main mechanisms that are thought to lead to the anti-inflammatory effects of regular exercise are a reduction in visceral fat mass (leading to a decrease in pro-inflammatory adipokines, e.g. TNF-α), an increased production of anti-inflammatory cytokines from contracting skeletal muscle (myokines; e.g. IL-6 leading to a subsequent rise in anti-inflammatory IL-10 and IL-1-RA) and a reduction of Toll-like receptor expression on monocytes and macrophages.[41]

Our results show that a high amount of weekly vigorous PA is associated with a great number of elevated cytokine levels in response to all four stimulants, indicating an immune system prepared for responding strongly in case of infection. PA is known to affect both innate and acquired immune response in various ways. As such, an increase in NK cell numbers and NK cell cytotoxicity, as well as a decrease in T cell functionality have been described in response to exercise.[22,24] An imbalance between Th1/Th2/Th17 cells and their control by Treg cells can play a crucial role in asthma development, while different phenotypes show distinct immunological patterns.[15,18-20] Type 2 inflammation is linked to the most common asthma phenotype, allergic asthma.[15,19] Type 2 cytokines (e.g. IL-4, IL-5, IL-9 and IL-13), which are activated by allergen exposure, can cause airway hyperresponsiveness by contraction of smooth muscles, mucus production, eosinophil activation and an induction of allergen-specific IgE by B-lymphocytes.[19,42] Those type 2 cytokines can also be secreted by innate lymphoid cells (ILC2), after stimulation by epithelial IL-25 and IL-33, which are generated by impaired airway epithelial cells in asthmatic subjects.[15,19,43]

Due to ethical limitations, immune response to PA in children has been poorly investigated.[24] In atopic individuals, PA may cause further Th2 polarization, leading to more severe allergic symptoms or exercise-related symptoms.[24] However, it has also been shown that exercising on a regular basis induced beneficial changes in allergic subjects, such as a reduction in pro-inflammatory cytokines (e.g. IL-4[44]) and a switch to a type 1 profile, which in turn may reduce allergic inflammation.[24] Studies in murine asthma models reported an enhancement of Treg responses to aerobic exercise.[25]

In the present study, we found some type 2 cytokines (IL-5, IL-9, IL-13 and CCL5) to be upregulated in asthmatic preschoolers with a high level of weekly PA, whereas IL-25 levels were significantly lower in more physically active individuals. The increase is not only found in type 2 cytokines, because regarding type 1- and type 17-related cytokines, IL-12B, IL-17, IL-27, TNF-α and CXCL10 were found to be upregulated in asthmatic preschoolers with high weekly PA, supporting the readiness to produce both type 1, type 2 and type 17 cytokines in response to various stimuli. These findings can be perceived as immunological fitness without any skew to a certain subtype observed by extensive PA.

Apart from the Th1/Th2 imbalance predominantly found in allergic asthmatics, a disequilibrium in Th17 and Treg cells has been noted in nonallergic asthmatic subjects with neutrophilic airway inflammation.[15,18]

IL-17, produced by Th17 cells, may be upregulated in these patients, while Treg functions are inhibited in children with asthma.[45,46] This Th17/Treg imbalance was shown to be closely associated with asthma severity and steroid-resistance.[15,47] The present study demonstrated IL-17A is upregulated in asthmatic preschoolers with high PA. As Th1 and Th17 cells, which produce IL-17A, are dominant in neutrophilic asthma,[15] it could be hypothesized that PA might not have a beneficial effect in every asthma phenotype. However, we also observed a decrease of IL-6 and IL-1β levels in highly physically active asthmatic preschoolers. IL-6 is required for Th17 differentiation and IL-1β promotes Th17 cell-dependent inflammation,[15] both leading to a disequilibrium of Th17/Treg cells (towards Th17). A fall in IL-6 and IL-1β levels may therefore positively impact the disbalance in such patients.

Longer TVA represent a longer indoor stay and less outdoor allergen and air pollutant exposure and a less physically active condition. Our results demonstrate an overall decrease in cytokine levels in asthmatic preschoolers with high daily TVA, indicating weaker immune responses to various stimuli (bacterial, viral or fungal) compared to subjects with less daily TV hours. However, the Th2-related cytokines IL-25, IL-33 and IL-13, all playing a major role in allergic asthma, were found to be downregulated in asthmatic children with high TVA. Since they are epithelial cytokines, longer stay indoors and less epithelial cell activation can be one of the reasons for this. It could therefore be argued that high TVA might have a positive impact on the epithelial cell alarmins that may be the initiators of type 2 inflammation. Furthermore, IFNγ, which has previously been found to be associated with non-eosinophilic asthma and steroid-resistant asthma,[15] was significantly downregulated in asthmatic preschoolers with high TVA.

While some of these results indicate a potential positive influence of TVA on immunological reactions in asthmatic preschoolers, our data also show that asthmatic subjects with low weekly PA and / or high daily TVA exhibited highly positively correlating proinflammatory cytokines under stimulated, but also unstimulated conditions, suggesting an overall proinflammatory state in those individuals. Furthermore, the association between a sedentary lifestyle and obesity is the be kept in mind. The worldwide increase in asthma prevalence occurred together with an increase in obesity and a sedentary lifestyle.[39] A number of studies reported an association between obesity and childhood asthma, however, the causality is not clear.[5,48]

Our study has a number of limitations. Firstly, the PreDicta cohort is moderate in size, however performing cell cultures in a standard way and measurement of many cytokines in all these patients and controls should be appreciated. The lack of objective criteria makes the diagnosis of asthma more difficult in preschoolers. In addition, parameters such as weekly PA or daily TVA were collected from questionnaires based on parental reports. Even though short-term parental reports were shown to be accurate,[49] recall bias might be a source of error. For example, strictly interpreting daily TV hours as sedentary behavior can lead to the wrong assumption that a subject with high daily TVA cannot simultaneously be vigorously physically active more than 3 times a week. It is unclear whether the differences observed in cytokine levels actually result from the physical activity status of the subjects. Many factors, such as genetics, type of asthma, asthma control, current medication, infections, immunizations, various exposures and diet can influence cytokine levels. For example, it can be well argued that the decrease in IL-25 levels in asthmatic children with high weekly PA might be due to their well-controlled asthma and the regular use of asthma medication. It has to be additionally considered and needs further studies whether children staying indoors with increased TVA are having less exposure to environmental pollutants and outdoor allergens. Furthermore, low PA and high TVA are more likely to be results of uncontrolled asthma, which will be addressed elsewhere. Lastly, it would have added great value to this study if BMI values had been assessed.

In conclusion, our results show that limited PA is likely the result of poor asthma control and that both PA and TVA possibly impact systemic immune response and immune and inflammatory thresholds in asthmatic preschoolers. Based on our findings, we recommend PA to be encouraged in asthmatic preschoolers, while good asthma control is essential. The fitness and readiness of the immune system to secrete cytokines is becoming more and more important, with the recent knowledge in COVID-19, for example, in timely release of anti-viral interferons.[50,51] It is also of great importance not to forget about the association of physical inactivity, poor physical cardiovascular fitness and obesity – all of which threatens a child's health and

well-being.

Abbreviations

AIRE Asthma Insights and Reality in Europe (study)

BMI Body Mass Index

CA State of California

CCL chemokine (C-C motif) ligand

CXCL chemokine (C-X-C motif) ligand

EIA exercise-induced asthma

FBS fetal bovine serum

GINA Global Initiative for Asthma

HEPES N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

IFN interferon

IL interleukin

MEM Minimum Essential Medium

MET metabolic equivalent

MyD88 myeloid differentiation primary response protein

NF-ϰB nuclear factor kappa-light-chain-enhancer of activated B cells

PA physical activity

PBMC peripheral blood mononuclear cell

PBS phosphate buffered saline

PEF peak expiratory flow

PHA phytohemagglutinin

Poly I:C polyinosinic:polycytidylic acid

PreDicta Post-infectious immune reprogramming and its association with persistence and chronicity of respiratory allergic diseases (study)

R848 Resiquimod

RNA ribonucleic acid

RPMI Roswell Park Memorial Institute Medium

SD standard deviation

TNF-α tumor necrosis factor alpha

TLR Toll-like receptor

TV television

TVA television attendance

WHO World Health Organization

Zymo zymosan

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Tables

Table 1. Patient characteristics, allergic comorbidities, asthma control, physical activity and TV attendance in asthmatic and healthy preschoolers.

Patient characteristics	**Healthy controls (n = 53)**	**Asthmatic patients (n = 140)**	**P value**
Gender (F/M)	31/22	58/82	ns
Age (y) mean ± SD	5.0 ± 0.8	5.3 ± 0.7	ns
Wheezer (yes), n (%)	0 (0%)	140 (100%)	**<.0001**
Atopic diseases (yes), n (%)			
Allergic rhinitis, n (%)	0 (0%)	89 (64%)	**<.0001**
Atopic dermatitis, n (%)	0 (0%)	73 (52%)	**<.0001**
Adverse food reaction, n (%)	0 (0%)	44 (31%)	**<.0001**

Patient characteristics	Healthy controls (n = 53)	Asthmatic patients (n = 140)	P value
Asthma control, n (%) Controlled Partly controlled Uncontrolled	– – –	74 (53%) 50 (36%) 16 (11%)	– – –
Vigorous physical activity (times per week), n (%) Never or occasionally Once/twice Three times or more	13 (25%) 10 (19%) 30 (57%)	42 (30%) 24 (17%) 75 (53%)	ns ns ns
TV attendance (hours per day), n (%) [?] 1 h 1-3 h [?] 3 h	25 (47%) 22 (42%) 6 (11%)	36 (26%) 82 (59%) 22 (16%)	**<.005 <.05** ns

Table 2. Asthma control, weekly physical activity and daily TV attendance in asthmatic preschoolers.

Asthma control	Controlled (n = 74)	Partially controlled (n = 50)	Uncontrolled (n = 16)	P value
Vigorous physical activity (times per week), n (%) Never or occasionally Once/twice Three times or more	15 (20%) 13 (18%) 46 (62%)	14 (28%) 11 (22%) 25 (50%)	12 (75%) 0 (0%) 4 (25%)	**<.0001** ns **<.05**
TV attendance (hours per day), n (%) [?] 1 h > 1 h	22 (30%) 52 (70%)	11 (22%) 39 (78%)	3 (19%) 13 (81%)	ns ns

Figure legends

Figure 1: Impact of asthma control on weekly physical activity (A) and TV attendance (B). ***A:*** Fraction of total (%) analysis of subjects with vigorous PA [?] 3 times a week, 1-2 times a week or never/occasionally physically active, grouped according to asthma control. B: Fraction of total (%) analysis of subjects with daily TV attendance > 1 h per day or [?] 1 h per day, grouped according to asthma control. *: $P < 0.05$ intergroup difference.

Figure 2 : Increased (A) and decreased (B) cytokines under different stimulations in asthmatic preschoolers. Violin plots with median and quartiles visualizing cytokine levels. Asthma n = 140, healthy n = 53. PBMCs were cultured with different stimulants for 48 hours. Cytokine levels were determined using a multiplex immunoassay. *: $P < 0.05$, **: $P < 0.01$ and ****: $P < 0.0001$ intergroup difference, respectively.

Figure 3 : Impact of physical activity and TV attendance on unstimulated cytokines in asthmatic (A, B) and healthy (C, D) preschoolers. Violin plots with median and quartiles visualizing

cytokine levels. Red arrows indicate increase or decrease of cytokine levels with higher PA or TVA. A: Asthma, physical activity: n /o (n = 42), 1-2 / w (n = 24), [?] 3 / w (n = 75). B: Asthma, TV attendance: [?] 1h / d (n = 36), 1-3h / d (n = 82), [?] 3h / day (n = 22). C: Healthy, physical activity: n /o (n = 13), 1-2 / w (n = 10), [?] 3 / w (n = 30). D: Healthy, TV attendance: [?] 1h / d (n = 25), 1-3h / d (n = 22), [?] 3h / day (n = 6). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ and ****: $P < 0.0001$ intergroup difference, respectively. n / o (never or occasionally), 1-2 / w (once or twice per week), [?] 3 / w (3 or more times per week), [?] 1h / d (less than one hour per day), 1-3h / d (one to three hours per day), [?] 3h / day (3 or more hours per day).

Figure 4 : **Impact of physical activity on PBMC cytokine response in asthmatic (A) and healthy (B) preschoolers.** Violin plots with median and quartiles visualizing cytokine levels. Red arrows indicate increase or decrease of cytokine levels with higher PA. A: Asthma, physical activity: n /o (n = 42), 1-2 / w (n = 24), [?] 3 / w (n = 75). B: Healthy, physical activity: n /o (n = 13), 1-2 / w (n = 10), [?] 3 / w (n = 30). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ and ****: $P < 0.0001$ intergroup difference, respectively. n / o (never or occasionally), 1-2 / w (once or twice per week), [?] 3 / w (3 or more times per week).

Figure 5 : **Impact of TV attendance on PBMC cytokine response in asthmatic (A) and healthy (B) preschoolers.** Violin plots with median and quartiles visualizing cytokine levels. Red arrows indicate increase or decrease of cytokine levels with higher TVA. A: Asthma, TV attendance: [?] 1h / d (n = 36), 1-3h / d (n = 82), [?] 3h / day (n = 22). B: Healthy, TV attendance: [?] 1h / d (n = 25), 1-3h / d (n = 22), [?] 3h / day (n = 6). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ and ****: $P < 0.0001$ intergroup difference, respectively. [?] 1h / d (less than one hour per day), 1-3h / d (one to three hours per day), [?] 3h / day (3 or more hours per day).

Figure 6: Correlations between cytokines in asthmatic and healthy preschoolers with low weekly physical activity (A, B) and high daily TV attendance (C, D). Spearman correlation heat maps visualizing correlations between measured cytokines in both asthmatic (A, C) and healthy (B, D) preschoolers. Red and blue squares indicate positive (0 to +1) and negative (0 to -1) correlations, respectively. E: Spearman correlation scatter plots showing significant correlations between cytokines.