

Received Date : 22-Apr-2014

Accepted Date : 27-Oct-2014

Article type : Original Article: Airway Diseases

Editor : Marek Sanak

TITLE PAGE

Title

Damage Associated Molecular Pattern and innate cytokine release in the airways of competitive swimmers

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/all.12540

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Author contributions

Recruiting of the patients and performing of the experiments: SS, VH, LVG, GM, ED, AK, SA, VV

Analysis and interpretation: SS, VH, LVG, GM, KP, JC, PH, LD, DB

Drafting the manuscript for important intellectual content: SS, VH, LVG, GM, KP, JC, PH, LD, DB

Keywords

Induced sputum; elite athlete; uric acid; Clara Cell protein-16; exercise-induced bronchoconstriction; eucapnic voluntary hyperventilation

Acknowledgements

We are very grateful to Lode Grossen and Kim Van Malderen of the elite sport high school for their cooperation to the project.

ABSTRACT

Background: Daily intensive exercise by elite athletes can result in exercise-induced asthma especially in elite swimmers and this may be linked to epithelial damage.

Objective: To study airway epithelial damage and release of damage associated molecular patterns (DAMPs) after intensive exercise in elite athletes and controls.

Methods: We recruited competitive swimmers (n=26), competitive indoor athletes (n=13) and control subjects (n=15) without any history of asthma. Lung function was measured before, immediately after and 24 hours after a 90-minute intensive exercise protocol. Sputum induction was performed at baseline and 24 hours after exercise. Exercise-induced bronchoconstriction (EIB) was assessed by the eucapnic voluntary hyperventilation test.

Results: Baseline sputum uric acid, High Mobility Group Box-1, CXCL8 mRNA, sputum neutrophils and serum Clara Cell protein-16 (CC-16) were significantly higher in competitive swimmers compared to control subjects. Intensive swimming for 90 minutes resulted in an increase of sputum IL-1 β , IL-6 and TNF mRNA in competitive swimmers, and of sputum IL-6 mRNA and sputum neutrophils in controls. Although all participants were asymptomatic, seven competitive swimmers, one indoor athlete and one control subject met the criteria for EIB.

Conclusion: Our findings show that intensive training combined with exposure to by-products of chlorination induces airway epithelial damage in competitive swimmers. This is associated with increased damage associated molecular patterns, innate cytokine release and neutrophilic airway inflammation.

TEXT

Introduction

Elite athletes daily perform intensive physical exercise. A high prevalence of asthma and exercise-induced bronchoconstriction (EIB) has been reported in elite athletes (1,2). In particular swimmers have a high risk for developing asthma (3). Competitive swimmers have the highest prevalence of airway hyperreactivity (up to 76%) among elite athletes (4).

Mechanical stress of (sustained) extreme breathing during training in combination with environmental conditions in which training is performed, may lead to airway epithelial cell damage and mediator

secretion that could initiate or increase inflammatory processes in the airways, in the end leading to asthma (5,6). Bronchial epithelial cells have indeed been shown to be released in higher amounts into sputum (epithelial shedding) of cold air athletes (e.g. cross-country skiers) and swimmers (7). Serum Clara Cell protein 16 has been used as a marker for disruption of the epithelial layer (8). Additionally, swimmers had higher sputum eosinophil percentages compared to control individuals (7) and those with airway hyperreactivity showed increased sputum neutrophil percentages 24 hours after a one-hour training session compared to baseline levels (9).

Unlike other elite athletes, swimmers are exposed to chlorine and chlorination by-products, such as trichloramine. Trichloramine in the air of indoor swimming pools may cause damage to the airways (10). In mice, it was shown that instillation of hypochlorite prior to ovalbumin induced airway hyperreactivity as a result of neuroimmune interactions (11).

Damaged or dying cells release Damage Associated Molecular Patterns (DAMPs) or danger signals: uric acid, High Mobility Group Box-1 (HMGB-1), Adenosine-5'-Triphosphate (ATP) and others (12). Mice exposed to uric acid crystals develop airway inflammation that is dependent on IL-1/IL-1R signalling (13). In asthmatic patients, uric acid is increased in broncho-alveolar lavage fluid after allergen challenge (14). We therefore hypothesize that intensive training induces DAMP secretion in the airways of elite athletes, which in turn induces airway inflammation.

In the present study we investigated sputum DAMP and cytokine expression in relation to markers of airway inflammation in competitive swimmers, competitive indoor athletes and control subjects before and after an intensive training session.

Methods

Subjects

Competitive swimmers and competitive indoor athletes (basketball or volleyball players) between 14-25 years of age were recruited among Belgian teams performing at national and international level (additional information: see Supporting Methods). Control subjects were required to be able to swim for 90 minutes and should not perform sports for more than 4 hours a week (2 hours sports at school and additional 2 hours sports at recreational level were allowed).

All subjects were evaluated before, immediately after and 24 hours after an intensive exercise session (swimming for swimmers and control subjects, an indoor training session for indoor athletes) of 90 minutes. The intensive exercise in all subjects consisted of a 60-minute protocol at submaximal level (70-80% of maximal heart rate (HR_{max})) followed by a 30-minute protocol consisting of progressively increasing levels of exercise to maximal heart rate (>80% of HR_{max}). Individuals with a history of asthma and who are currently taking anti-asthmatic drugs (inhaled β_2 -agonists and/or inhaled steroids) were excluded (Table 1). Baseline characteristics are presented in Table 2. The study was approved by the institutional review board and registered at clinicaltrials.gov (NCT01942096). Details on clinical evaluation and lung function evaluation can be found at the Supporting Methods.

Lung function evaluation

Eucapnic voluntary hyperventilation test (on a separate day) was used to assess exercise-induced bronchoconstriction (EIB) according to previous standards (15,16). The EVH test was considered positive if the fall in $FEV_1 \geq 10\%$ at one of the time points after the test.

A fall in FEV₁ after the intensive training session compared to baseline was assessed immediately and 24 hours later. Exercise-induced bronchoconstriction was suspected if there was a drop in FEV₁ ≥10% immediately after training. Reversibility of FEV₁ 15 minutes after inhalation of 400 µg of salbutamol was assessed 24 hours after exercise.

Sputum induction

Induced sputum was obtained by nebulizing a hypertonic salt solution (3%, 4% and 5% NaCl for 7 minutes) as previously described (17). Sputum samples were processed by selected plug method as previously described (see Supplementary Methods) (17,18).

Analysis of induced sputum and blood analysis

A selected panel of cytokines known to be involved in asthma was analysed (17,19–21). This includes: Th1 (IFN-γ), Th2 (IL-4, IL-5), Th22 (IL-22), epithelial-derived (IL-25, IL-33, TSLP) and innate (IL-1β, IL-6, CXCL8, TNF) cytokines. Sputum cytokine mRNA levels were measured RT-PCR (17,19,20). Primer and probe sequences were listed in the supporting information. Sputum uric acid levels were quantified by Amplex Red Uric Acid/Uricase Assay kit from Life Technologies (Carlsbad, USA) according to the manufacturers' protocol. Samples were diluted 1/5 to reduce interference of DTT in the sputum.

Sputum myeloperoxidase (MPO) levels (Hycult Biotech, Uden, The Netherlands), sputum HMGB-1 levels (Mybiosource, San Diego, USA) and serum Clara Cell protein-16 (CC-16) (Biovendor, Modrice, Czech Republic) were quantified by ELISA according to the manufacturers' protocol.

Statistical analysis

Details on statistical analyses can be found at the Supporting Methods.

Results

Exercise-induced bronchoconstriction

Twenty-three percent of swimmers (6/26; $\text{Chi}^2=1.2$, $p=0.14$) had a positive eucapnic hyperventilation (EVH) test ($\geq 10\%$ fall in FEV_1) compared to none of the indoor athletes (0/13) and one of the controls (Figure 1A). Two swimmers had $\geq 10\%$ fall in FEV_1 immediately after intensive exercise, of whom one also had a positive EVH test (Figure 1B). A FEV_1 increase $\geq 12\%$, 15 minutes after inhalation of 400 μg salbutamol, was found in 7 swimmers at 24 hours after exercise, but in none of the indoor athletes or control subjects (data not shown). However, these swimmers had decreased FEV_1 levels 24 hours after exercise compared to baseline.

Baseline markers of airway inflammation and DAMPs

None of the control individuals had sputum eosinophil counts $\geq 3\%$ or neutrophil counts $>40\%$. Baseline sputum eosinophil ($p=0.02$; Figure 2A) and neutrophil ($p=0.008$; Figure 2B) counts were significantly higher in swimmers compared to controls. Baseline sputum neutrophils ($p=0.0005$; Figure 2B) were also significantly higher in indoor athletes compared to controls. Five (19%) swimmers and three (23%) indoor athletes had sputum eosinophils $\geq 3\%$. Nine (35%) swimmers and seven (54%) indoor athletes had sputum neutrophils $>40\%$ (Figure 2B). No significant differences in baseline sputum MPO levels, representing activation of neutrophils, were observed between swimmers, indoor athletes or controls (data not shown). Swimmers had significantly higher baseline sputum CXCL8 mRNA levels compared to controls ($p=0.006$; Figure 2C).

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Baseline sputum IFN- γ mRNA levels (Th1 cytokine) were significantly higher in swimmers ($p=0.031$; Figure E1 in the supporting information) and indoor athletes ($p=0.039$; Figure E1) compared to controls. Baseline sputum IL-1 β , IL-4, IL-5, IL-6, IL-25 and TSLP mRNA levels were not significantly different between swimmers, indoor athletes and controls (data not shown). Baseline sputum uric acid and HMGB-1 levels were higher in swimmers ($p=0.009$ and $p=0.0003$) but not in indoor athletes ($p=0.24$ and $p=0.22$) compared to controls (Figure 2D/E).

Effect of intensive exercise on airway inflammation and cytokine release

Sputum eosinophil and neutrophil counts were not altered by intensive exercise in swimmers and indoor athletes (data not shown). In contrast, sputum neutrophils were higher after intensive exercise in controls ($p=0.02$; data not shown). Intensive exercise did not induce a significant increase of sputum uric acid (UA) or HMGB-1 in any of the groups (data not shown). However, swimmers with a positive EVH test showed a significant increase in sputum uric acid levels 24 hours after swimming compared to baseline (Figure E2a). Sputum IL-1 β mRNA levels were significantly increased in swimmers 24 hours after swimming ($p=0.01$; Figure 3A) but not in indoor athletes (Figure E3a) or controls (Figure E3b). Sputum IL-6 ($p=0.01$; Figure 3B) and TNF ($p=0.04$; Figure 3C) mRNA levels also significantly increased after intensive exercise in swimmers and sputum IL-6 mRNA levels ($p=0.01$; Figure E4b) significantly increased in controls. No significant induction of epithelial cell derived cytokines (IL-25, IL-33 and TSLP) was observed in sputum 24 hours after sport-specific training in any of the three groups (data not shown).

Serum biomarker for epithelial damage

Baseline serum Clara cell protein-16 was significantly higher in swimmers ($p=0.001$; Figure 5A) and indoor athletes ($p=0.02$; Figure 5A) compared to controls. No significant induction of serum CC-16

after intensive exercise was seen in swimmers or controls (data not shown). Surprisingly, serum CC-16 levels decreased after intensive exercise in indoor athletes ($p=0.005$; data not shown).

Discussion

In the present study, we found that 27% of competitive swimmers, without history of asthma and who were not on regular treatment with anti-asthmatic medication, had either a positive eucapnic voluntary hyperventilation (EVH) or a positive field exercise test. Twelve competitive swimmers were a priori excluded from the study because of history of asthma and regular treatment with anti-asthmatic medication. Most previous studies assessed airway hyperreactivity in elite athletes by histamine or metacholine challenge. Elite swimmers were shown to have the highest prevalence of airway hyperreactivity (up to 76%) among elite athletes (4). Histamine or methacholine challenge tests however, although being helpful for the diagnosis of asthma, are not specific for the diagnosis of EIB. Recently, an official ATS (American Thoracic Society) clinical practice guideline for exercise-induced bronchoconstriction was published (15), recommending serial lung function measurements after exercise or hyperpnea challenge (EVH). Both tests have advantages and disadvantages. The field exercise test can be performed in the environment in which exercise typically occurs but is less sensitive compared to the EVH test to detect EIB in athletes (22). On the other hand, the EVH test can be performed in a standardized manner but is laboratory dependent and can only be performed in specialized centres (23). A study by Martin and colleagues showed that 72% of pool-based athletes with symptoms of exercise-induced asthma (not taking inhaled corticosteroids) had a positive EVH test compared to 39% of non-pool based athletes (24). The lower prevalence of EIB in our study might be due to the a priori selection of non-asthmatic swimmers. In a study of adolescent (12-16 years of age) elite swimmers who stopped inhaled steroids 4 weeks prior to the study, 27.6% of swimmers had a positive EVH test (25).

Besides these signs of airway hyperreactivity and EIB, we found neutrophilic airway inflammation (>40% sputum neutrophils) in both swimmers (n=9) and indoor athletes (n=7) at baseline. Thus, sustained intensive training, irrespective of exposure to by-products of chlorination, induces neutrophilic airway inflammation. In line with these results, we previously reported increased sputum neutrophils, IL-17A and MPO levels after exposure to intensive exercise, cold air and high altitude (expedition to the Aconcagua mountain) in patients with asthma (26). In our present study, there was no further increase in sputum neutrophilia 24 hours after intensive exercise in swimmers or indoor athletes, while in control subjects we did notice a significant increase in sputum neutrophil counts 24 hours after swimming. These data suggest that an acute exercise protocol at a high level induces neutrophilic airway inflammation. We hypothesize that no further increase of sputum neutrophils in swimmers and indoor athletes is seen because of already high baseline levels.

Baseline sputum CXCL8 levels were significantly higher in swimmers compared to control subjects. This was not observed in indoor athletes. Local CXCL8 can be released in the airways by bronchial epithelial cells, fibroblasts and airway smooth muscle cells. We recently reported a highly significant positive correlation between sputum CXCL8 mRNA levels and sputum neutrophil counts in allergic and non-allergic patients (19). Although this correlation did not prove that neutrophils are attracted to the airways by local CXCL8, a cause-effect relationship seems plausible.

We were also able to demonstrate increased presence of Damage Associated Molecular Patterns in the sputum of the athletes, which may feature as early inducers of the pro-inflammatory cytokines. Bronchial epithelial cells as well as alveolar macrophages release DAMPs, which was shown in the airways of COPD subjects (27). Bougault and colleagues showed that bronchial epithelial cells in sputum are significantly higher in elite swimmers compared to controls at baseline suggesting epithelial shedding in elite swimmers (28). A damaged airway epithelium could then result in release of DAMPs in the airways of athletes, which was confirmed by our data. Sputum uric acid and High

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Mobility Group Box (HMGB)-1 were higher at baseline in swimmers compared to controls. Sputum HMGB-1 has been proposed as a marker for neutrophilic airway inflammation (29), which may help to explain the higher sputum neutrophil count in swimmers compared to controls. HMGB-1 may induce the transition of epithelial cells to mesenchymal structures (30). In line with this, increased airway remodelling has been shown in elite swimmers compared to control subjects (31). Remarkably, uric acid was induced after intensive swimming only in swimmers with a positive EVH test. No further increase in sputum uric acid or HMGB1 levels could be observed in any of the total groups after exercise, but an increase in IL-1 β mRNA levels (downstream to uric acid) was found in swimmers 24 hours after the training session. It has previously been shown that uric acid is released upon allergen challenge in allergic asthmatics and contributes to the mounting of Th2 immunity (14). Release of uric acid causes activation of NALP3 inflammasome and cleavage of pro-IL-1 β to IL-1 β via caspase 1 (32). IL-1 β then creates a pro-inflammatory environment with release of IL-6 and chemokines (like CXCL8) facilitating recruitment of neutrophils and a Th17 response (32). We also found higher baseline serum CC-16 levels in swimmers and indoor athletes compared to controls. Unexpectedly, serum CC-16 levels decreased in indoor athletes 24 hours after intensive exercise. We however cannot explain this finding. Serum Clara Cell protein-16 has been proposed as a biomarker for airway epithelial damage (8). Thus, epithelial damage is induced by sustained intensive exercise, irrespective of exposure to by-products of chlorination. This is consistent with results of Chimenti and colleagues who showed increased serum CC-16 levels and bronchial epithelial cells after a half-marathon in non-asthmatic amateur runners (33). However, only few bronchial epithelial cells were detected in sputum samples of our study. This might be explained by the time point of sampling that was chosen for our study and which was not optimal to detect epithelial damage at the cellular level.

A limitation of the current study is the low number of individuals analysed in the study. A strong selection was however made to include athletes in the study. We therefore cannot be certain if lack of significance is due to small sample sizes or represent a real lack of difference between the groups.

In conclusion, a quarter of elite swimmers without history of asthma were found to have exercise-induced bronchoconstriction. Elite swimmers had higher levels of sputum DAMP molecules (uric acid and HMGB-1), innate cytokines (IL-1 β , IL-6 and TNF) and neutrophils in the airways. Also, the biomarker Clara Cell protein-16 was found to be higher in serum of swimmers and indoor athletes compared to controls. We thus suggest that intensive exercise, especially in combination with exposure to by-products of chlorination, induces damage of the airway epithelium, thereby releasing DAMP molecules and activating a cascade of inflammation resulting in a recruitment of predominantly neutrophils in the airways.

Funding

The study was funded by the Climbing for life research fund, Belgium, the Interuniversity Attraction Poles Program-Belgian State-Belgian Science Policy- project P7/30 and the Study Centre for Allergy Projects (SCAP), Haarlem, The Netherlands. Lieven Dupont and Dominique Bullens are recipients of a senior researcher fellowship from the Fund for Scientific Research Flanders (FWO).

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- Accepted Article
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FIGURE LEGENDS

Figure 1. Exercise-induced bronchoconstriction tests at baseline.

A. Competitive swimmers (n=24), competitive indoor athletes (n=13) and control subjects (n=11) performed eucapnic voluntary hyperventilation test for 6 minutes at a target rate of 85% of Maximal Voluntary Ventilation (MVV). Spirometry was performed at baseline and 1', 5', 10' and 15' after MVV. The test was considered positive if there was a fall in $FEV_1 \geq 10\%$ on at least one time point.

B. Competitive swimmers (n=26), competitive indoor athletes (n=13) and control subjects (n=15) performed an intensive training session for 90 minutes. During the last 30 minutes, intensity was progressively increased to reach a maximum at the end of the test. Spirometry was performed at baseline and 1' after and the test was considered positive if there was a fall in $FEV_1 \geq 10\%$. Chi square test was used to compare proportions of patients with positive EVH test or intensive exercise protocol test.

Figure 2. Airway inflammation at baseline.

Sputum differential cell counts (eosinophils (A) and neutrophils (B)) were counted on cytospins. Sputum CXCL8 mRNA was measured on lysed sputum cells and normalized to β -actin (C). Sputum uric acid (D) and sputum HMGB-1 (E) was measured on sputum supernatant. Individuals with a positive eucapnic voluntary hyperventilation test were marked in black. Mann-Whitney test was used to compare swimmers and indoor athletes with controls.

Figure 3. Sputum innate cytokines at baseline and 24 hours after intensive training (swimming: swimmers and controls, basketball or volleyball: indoor athletes). Sputum IL-1 β , IL-6 and TNF mRNA was measured on lysed sputum cells and normalized to β -actin. Individuals with exercise-

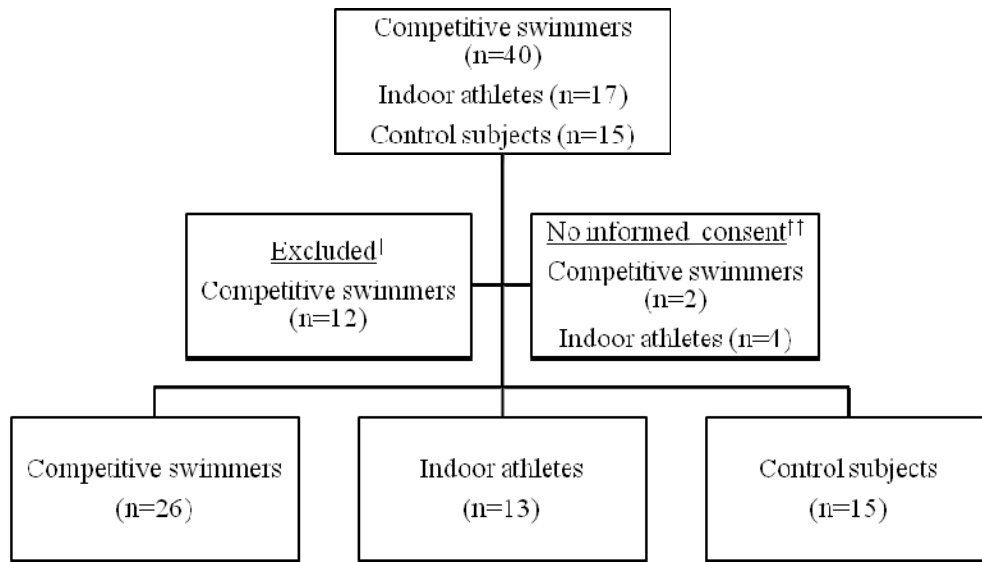
induced bronchoconstriction were marked in black. Wilcoxon signed rank test was used to compare results at two time points.

Figure 4. Serum Clara Cell protein-16 was measured at baseline in swimmers, indoor athletes and control subjects.

Individuals with exercise-induced bronchoconstriction were marked in black. Mann-Whitney test was used to compare swimmers and indoor athletes with controls.

TABLES

Table 1



Enrolment to the study.

[†]: Twelve swimmers were excluded because of regular treatment with β_2 -agonists (n=4) and/or inhaled steroids (n=8).

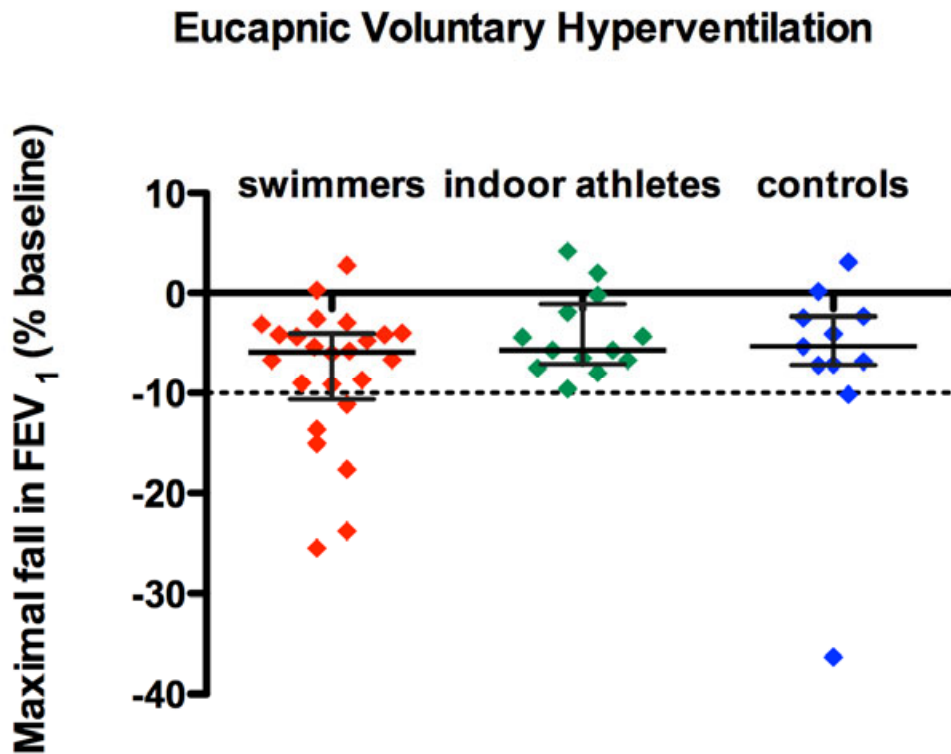
^{††}: Two swimmers and 4 indoor athletes denied informed consent.

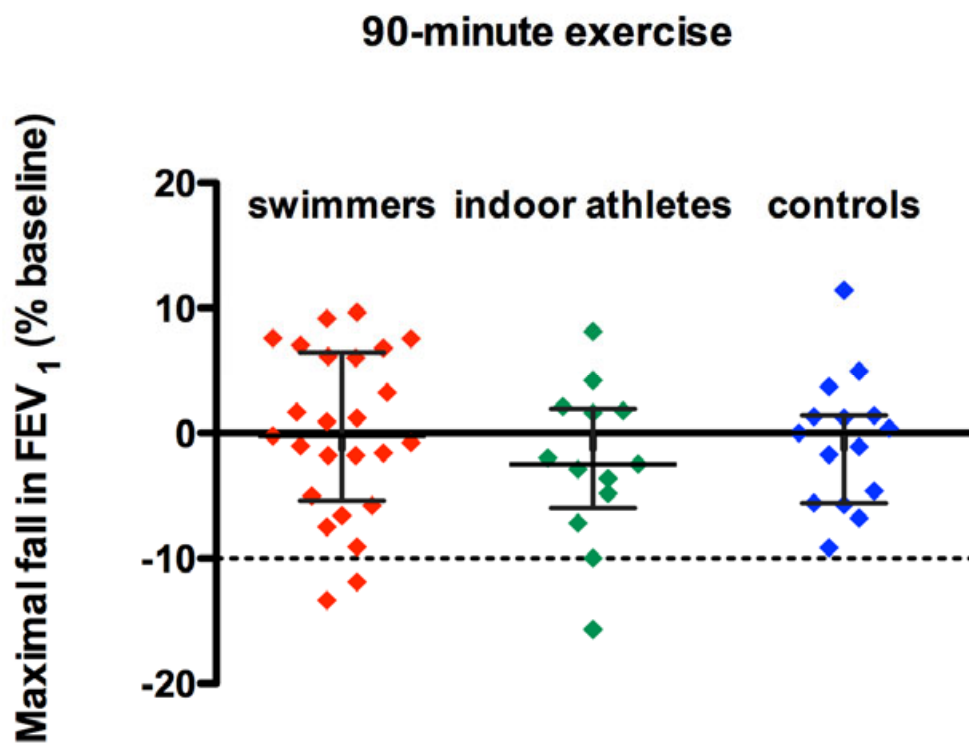
Table 2. Subject characteristics

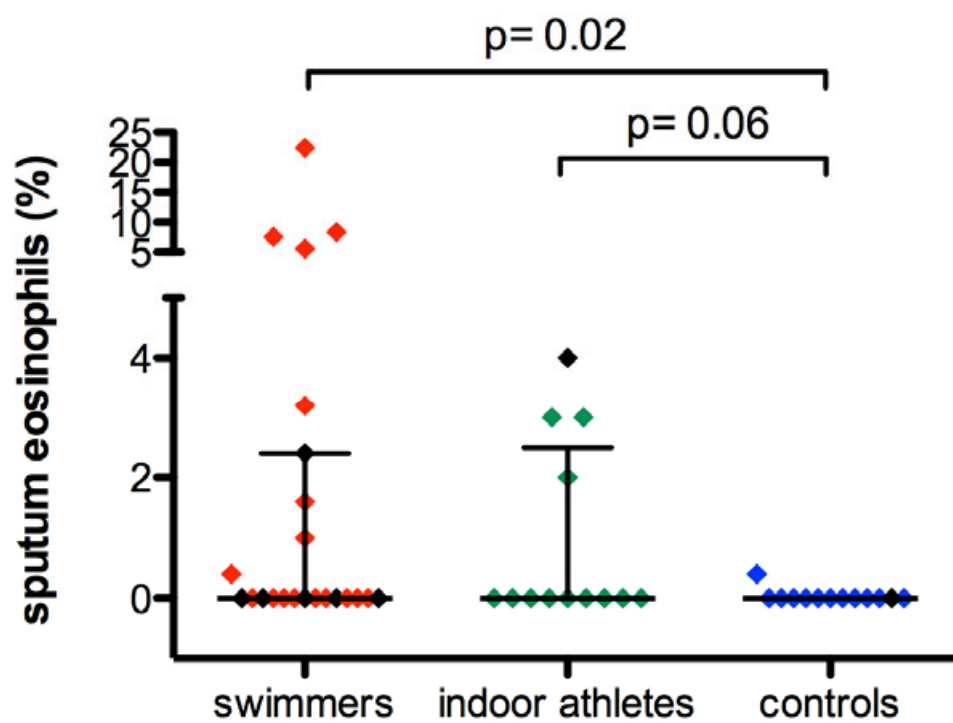
	Swimmers	Indoor athletes	Controls	P value
Number (n=)	26	13	15	
Age (years)	16.5 $\pm$ 2.2**	18.5 $\pm$ 1.8	19.2 $\pm$ 3.6	0.004
Gender (M / F)	16 / 10	8 / 5	7 / 8	0.61
Body Mass Index	21.0 $\pm$ 1.5	20.3 $\pm$ 1.8	22.1 $\pm$ 2.1	0.06

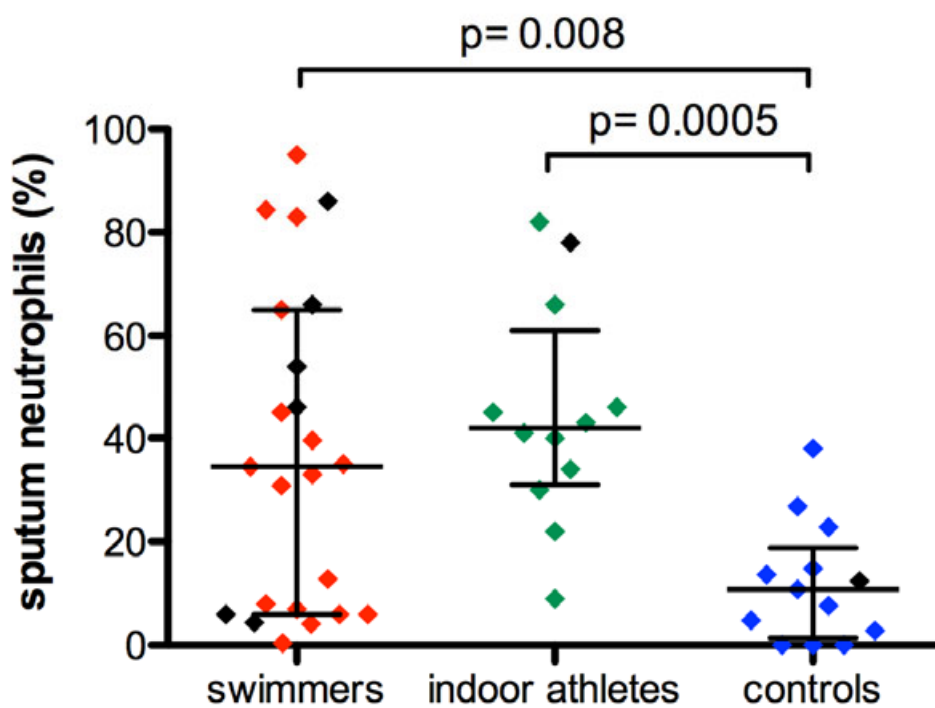
Atopy (n=)	5 (19%)	3 (23%)	4 (27%)	0.86
Training (years)	9.3 ± 2.0	9.5 ± 2.4	n.a.	0.40
Training / week (hours)	15.0 ± 3.7	14.8 ± 1.0	<4.0	0.24
FEV ₁ (L)	4.9 ± 1.0 ^{**}	4.5 ± 1.0	3.8 ± 1.1	0.01
FEV ₁ % predicted	108.2 ± 14.0	100.0 ± 20.0	98.8 ± 13.2	0.12
FVC (L)	6.3 ± 1.4 ^{***,†}	4.6 ± 1.3	4.6 ± 1.3	0.0002
FVC % predicted	119.8 ± 18.6 ^{***,‡}	99.7 ± 18.0	99.6 ± 13.5	0.0004

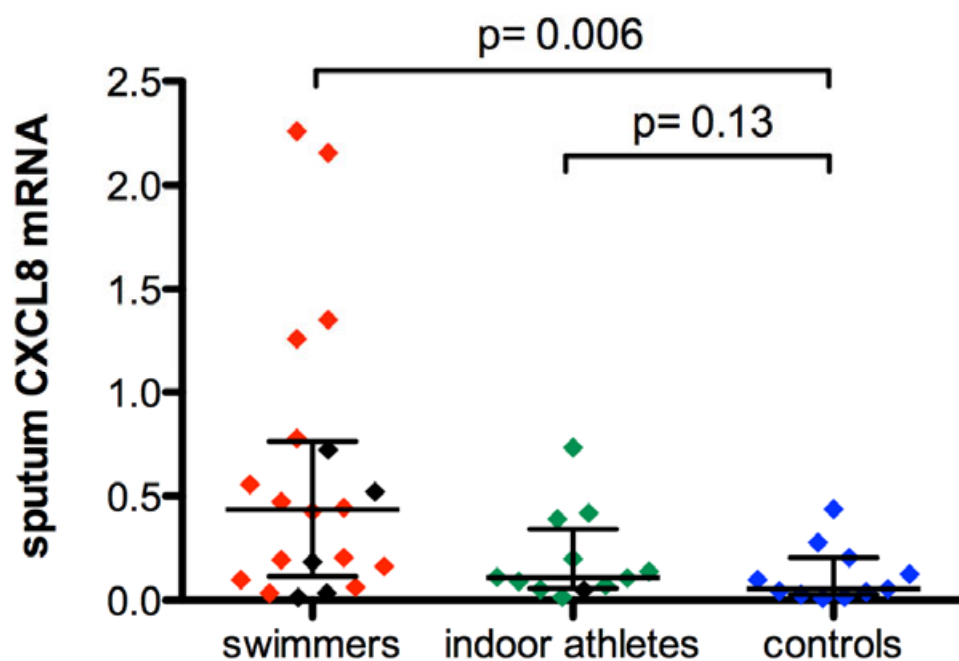
Patient is defined as atopic if skin prick tests were positive for at least one allergen. ^{**}: p<0.001 compared to controls, ^{***}: p<0.0001 compared to controls, [†]: p<0.01 compared to indoor athletes, [‡]: p<0.001 compared to indoor athletes, n.a.: not applicable.

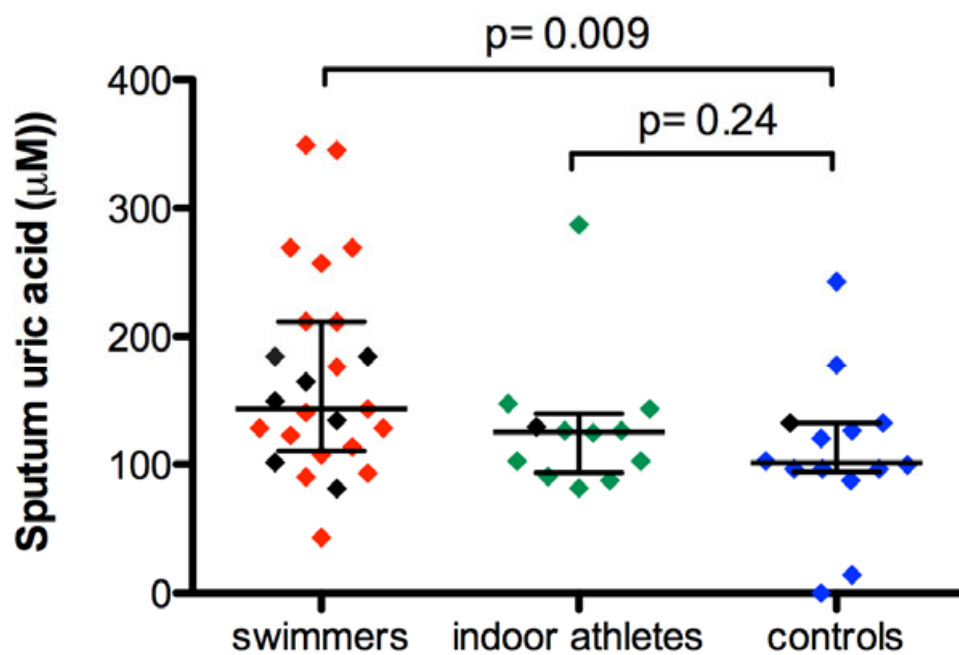


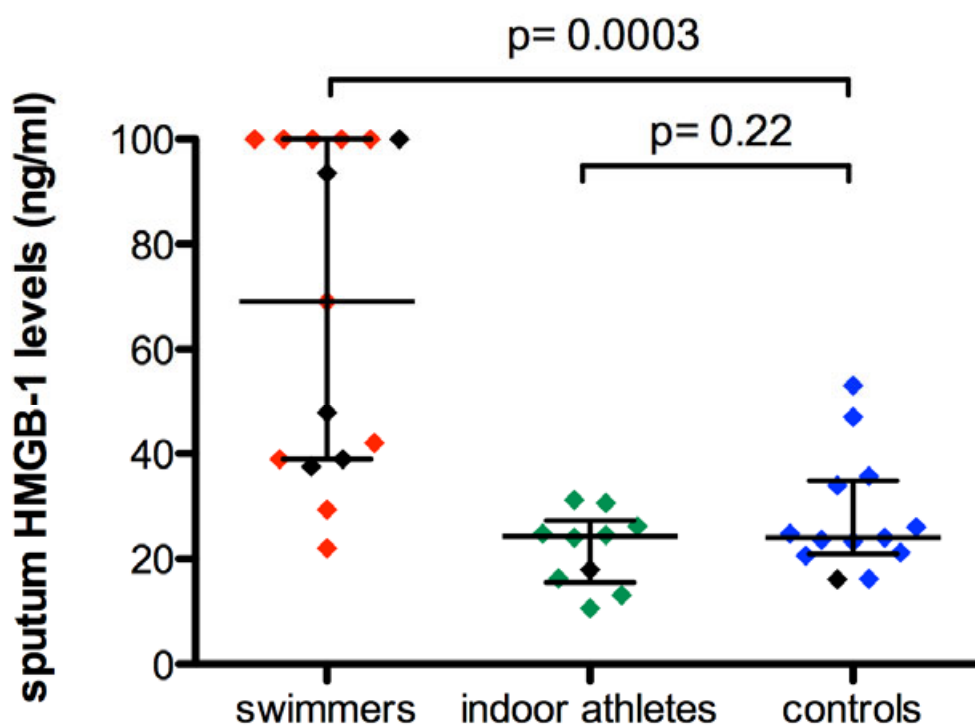


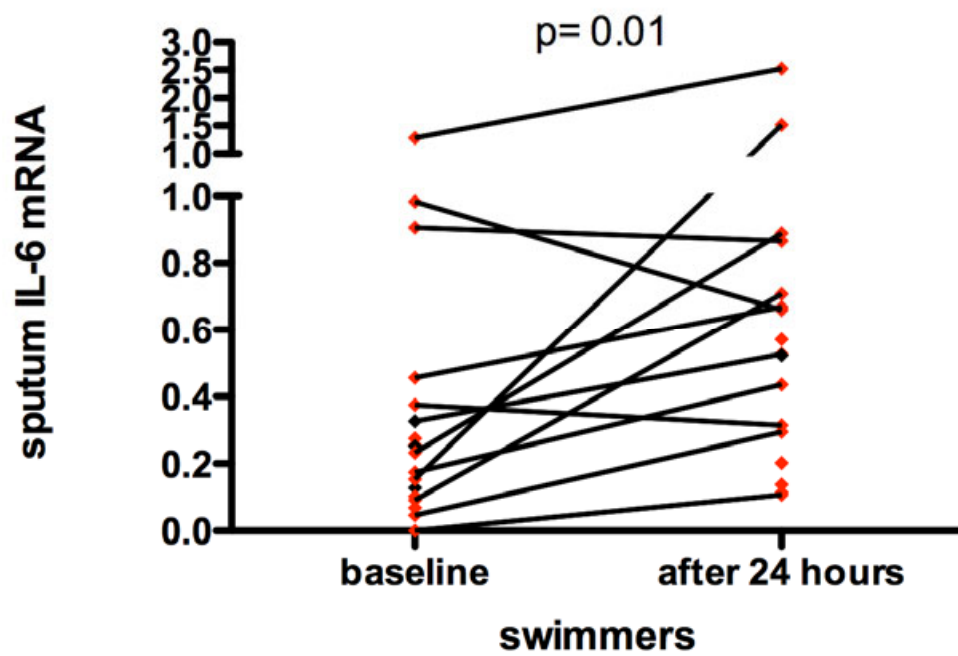
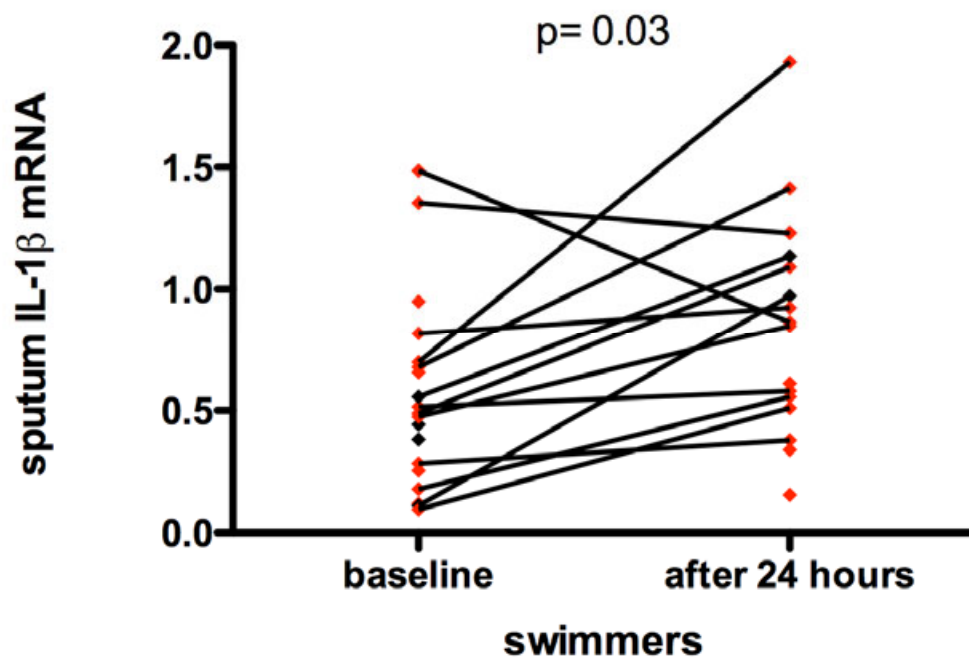


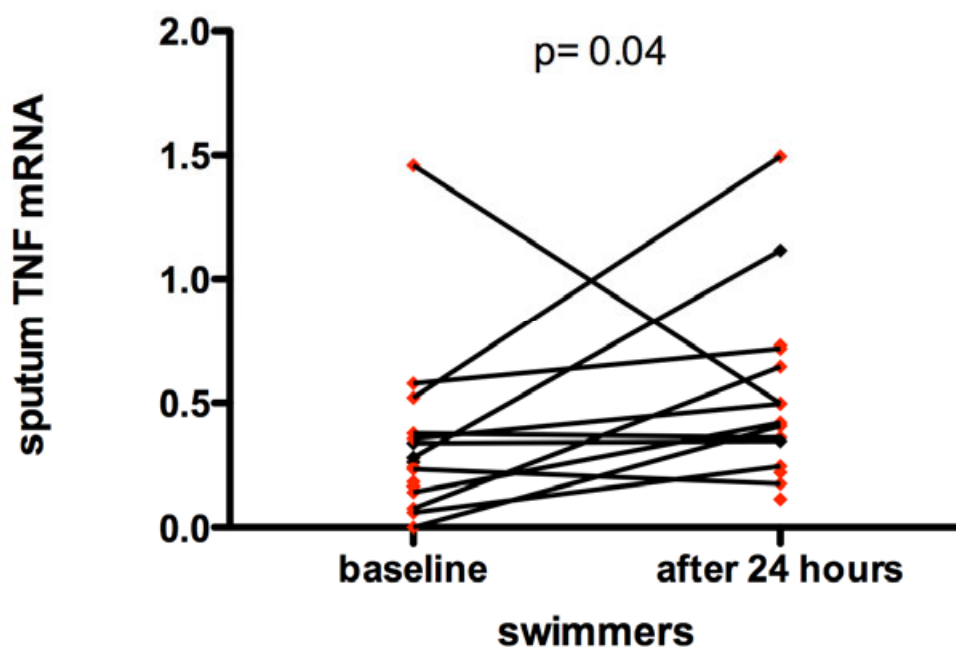


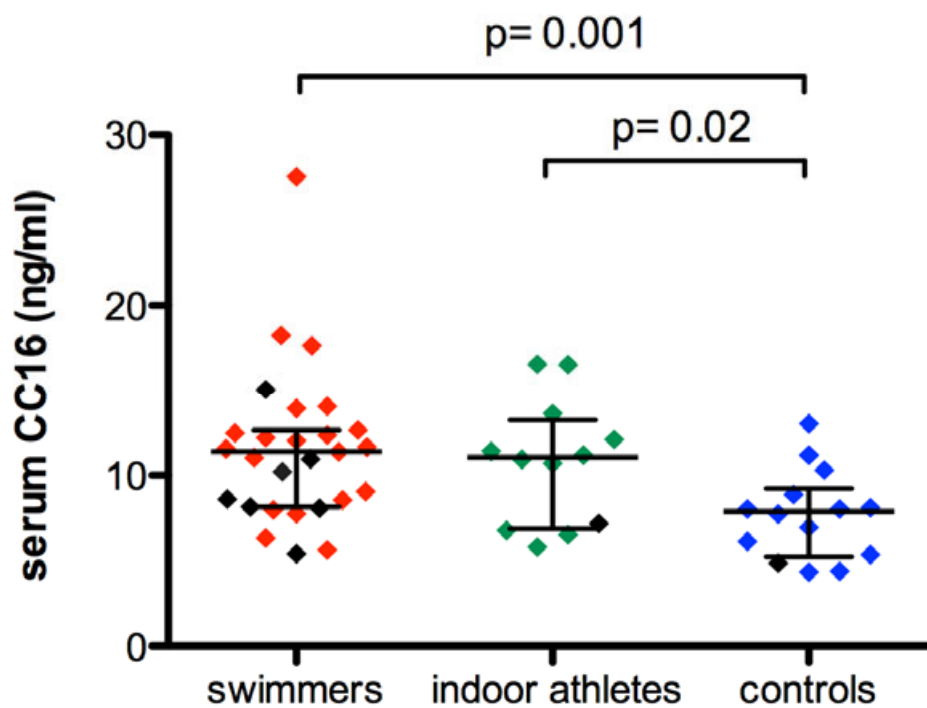












Supporting information

Title

Damage Associated Molecular Pattern and innate cytokine release in the airways of competitive swimmers

Authors

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Table S1.

	25% Percentile	Median	75% Percentile	Flemish regulations
Swimming pool water				
pH	7.18	7.27	7.33	7.0-7.6
free chlorine (mg/L)	0.93	1.10	1.28	0.5-3.0
bound chlorine (mg/L)	0.19	0.38	0.60	≤ 1.0
Temperature (°C)	27.50	27.90	28.18	≤ 32.0
Swimming pool air				
Trichloramine (mg/m ³)	0.18	0.28	0.38	0.5*
Temperature (°C)	27.50	27.80	28.50	
Relative humidity (%)	57.25	62.35	68.43	

*: No regulation in Flemish part of Belgium. World Health Organisation recommends a provisional value of 0.5 mg/m³.

Supporting Methods

Subjects

Two swimmers and 4 indoor athletes, who were invited for the study, did not give informed consent and were therefore not included. Swimmers, indoor athletes or control subjects with a prior diagnosis of asthma and active treatment with any anti-asthmatic medication (inhaled β_2 -agonists and/or inhaled steroids) were excluded. Competitive swimmers (n=26), competitive indoor athletes (n=13) and age-matched controls (n=15) were enrolled in the study (Table 1). Individuals in the control group performed the following sports at a recreational level: dancing (n=2), running (n=4), hockey (n=1), football (n=3), swimming (n=1) and basketball (n=1) or nothing (n=5).

Clinical evaluation

Dynamic lung volumes were measured with spirometry (Spirolab II, MIR, Rome, Italy). Allergy for house dust mite, grass pollen, tree pollen, weeds, pets and moulds was assessed by means of skin prick test (HAL Allergy, Leiden, The Netherlands). A patient was considered to be atopic if skin prick test was positive for at least one allergen.

Lung function evaluation

Eucapnic voluntary hyperventilation test was used to assess exercise-induced bronchoconstriction (EIB). In brief, subjects breathed a gas mixture (5% CO₂, 21% O₂ and 74% N₂) at a target rate of 85% of their maximal voluntary ventilation (MVV) per minute (assessed before the EVH test) for 6 minutes. The gas mixture was inhaled from a non-diffusing gas bag (Hans Rudolph, Shawnee, USA) and through a two-way T-shape

valve (Hans Rudolph, Shawnee, USA). Spirometry was performed at 1, 5, 10 and 15 min after the EVH challenge.

Sputum induction

Induced sputum was obtained by nebulizing a hypertonic salt solution (3%, 4% and 5% NaCl for 7 minutes). Sputum samples were processed by selected plug method. A dithiothreitol (DTT)-containing buffer was added to the sputum plugs to release cells from the mucus followed. The sputum sample was incubated on the roller for 15 minutes. A bovine serum albumin (BSA)-containing buffer was then added and incubated for 5 minutes. After filtering the sample through a 70µm filter, the cell suspension was centrifuged and total number of sputum cells was determined. Sputum supernatant was aliquoted and stored at -80°C. Cytospins (Shandon cytocentrifuge) were prepared from the pellet for differential cell counts and the remaining cells were lysed for mRNA analysis.

Primer and probe sequences

The primers and probes for β -actin, IL-4, IL-5, IL-6, CXCL8, IL-25, IL-33, IFN- γ and TSLP have been reported (Truyen et al, Thorax, 2006; Seys et al, Clin Exp Allergy, 2013). All primers and probes were purchased from Applied Biosystems or Eurogentec S.A. The primer and probe sequence for IL-1 β were designed with Primer Express (Applied Biosystems):

IL-1 β FW 5' ttg ctc aag tgt ctg aag cag c 3'

IL-1 β RV 5' caa gtc atc ctc att gcc act g 3'

IL-1 β TP 5' tac ctg agc tcg cca gtg aaa tga tgg 3'

Analysis of swimming pool water and air

Free chlorine and bound chlorine in swimming pool water was quantified by a standardized colorimetric test using DPD (N,N- diethyl-p- phenylenediamine) (lab Derva, Beringen, Belgium). Temperature, pH of swimming pool water and temperature, relative humidity of swimming pool air were analyzed (see Table E1 in the online data supplement). Trichloramine (by-product of chlorination) in air was analysed according to the reference method developed by Héry et al. (31). Requisite sampling cassettes were prepared and analyzed by lab Derva (ISO 17025 qualified, Belac). In brief, trichloramines are sampled from the air using an air pump connected to a series of filters in a filter holder. The first filter is a Teflon filter to avoid chlorides present in the air from reaching the final filter. The final filter used for the quantitative analysis of the trichloramines is treated with a solution of sodium carbonate and diarsenic dioxide to transfer the trichloramines to chlorides. Air samples were pumped at a 2 L/min flow rate for 120 min through the cartridges 1.5m above the water surface. All pumps were calibrated each morning prior to sampling.

The chlorides on the final filter are analysed by ion chromatography and recalculated to the concentration of trichloramine per m³ air. The LOD (limit of detection) was 50 $\mu\text{g}/\text{m}^3$.

Statistics

Statistical analyses were performed with Graphpad Prism V for Macintosh (Graphpad Software Inc., San Diego, USA) by use of Kruskal-Wallis, Dunn's multiple comparison

test (as post-test) and Mann-Whitney U test when appropriate. ANOVA or T-test was performed if data were normally distributed. Paired data were analysed by paired T-test or Wilcoxon signed rank test where appropriate. Normality was analyzed prior to between group analyses by Shapiro-Wilk test. A difference was considered to be significant when $p < 0.05$.

Supporting Figures

Figure E1.

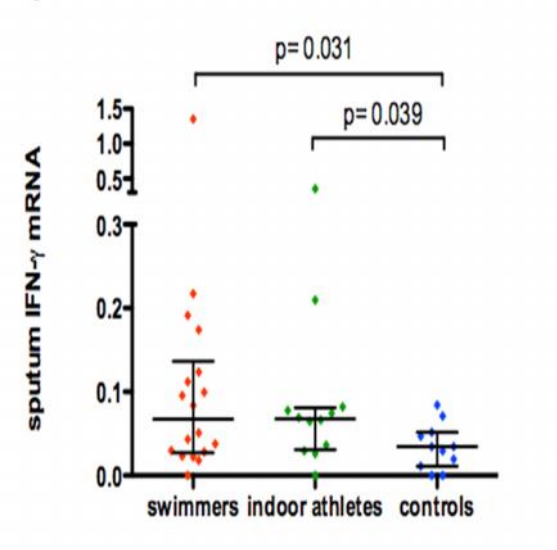


Figure E2a.

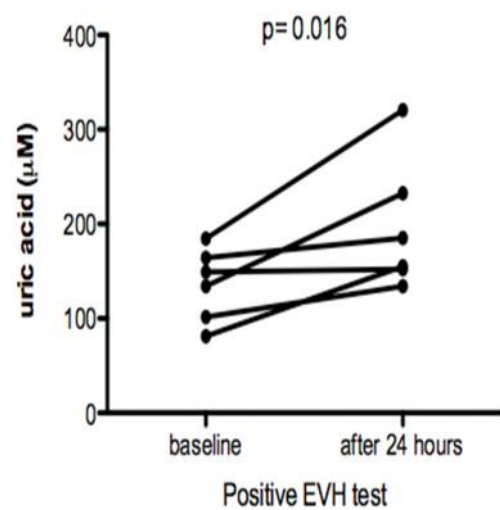


Figure E2b.

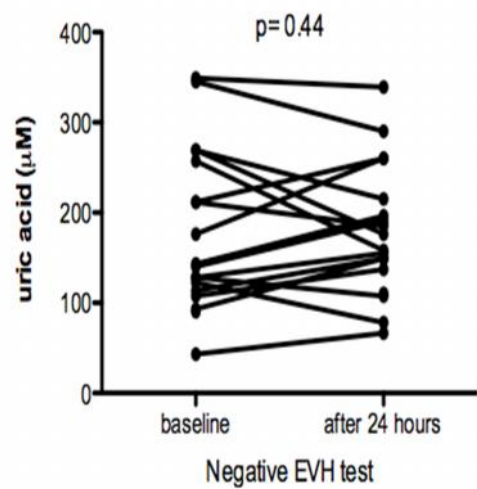


Figure E3a.

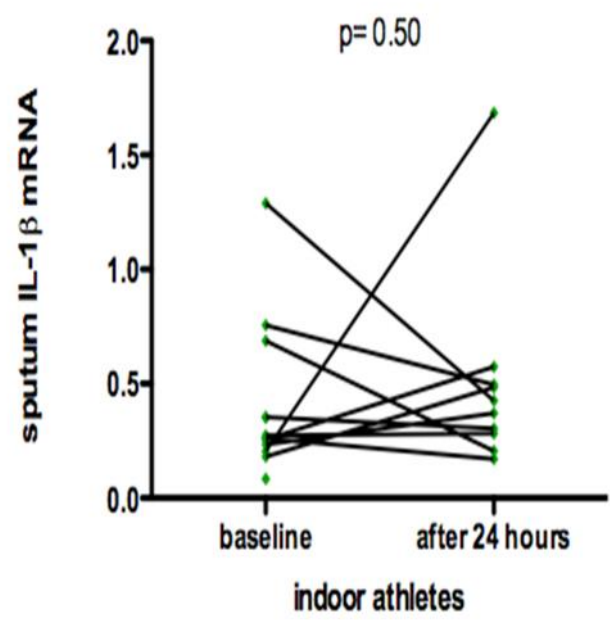


Figure E3b.

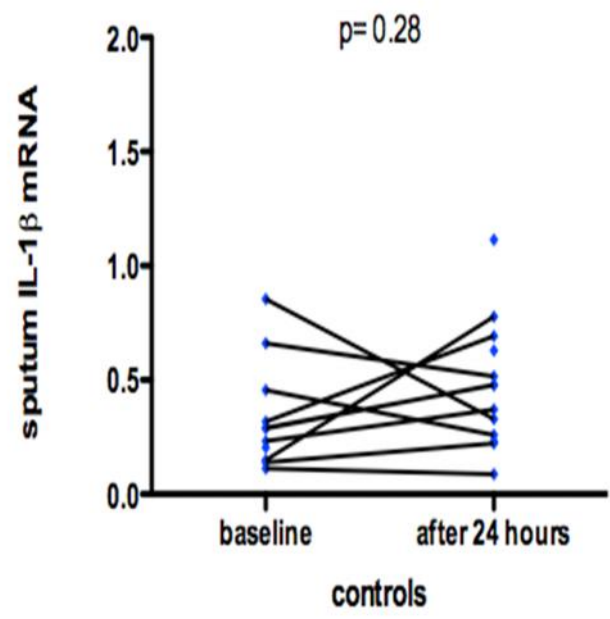


Figure E4a.

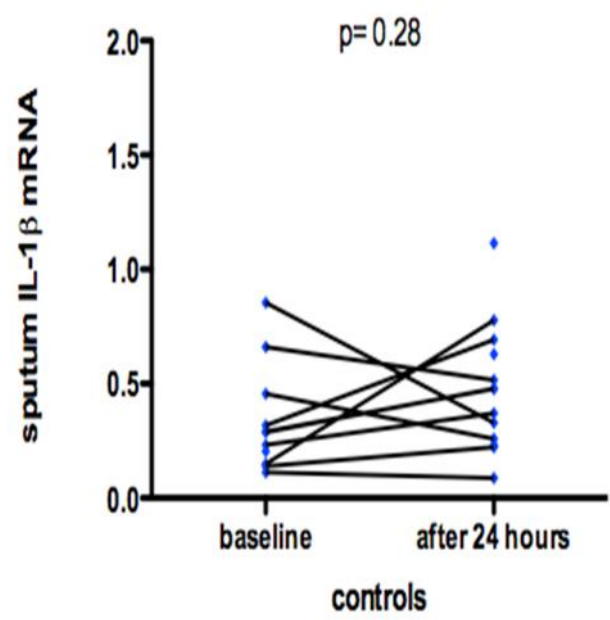


Figure E4b.

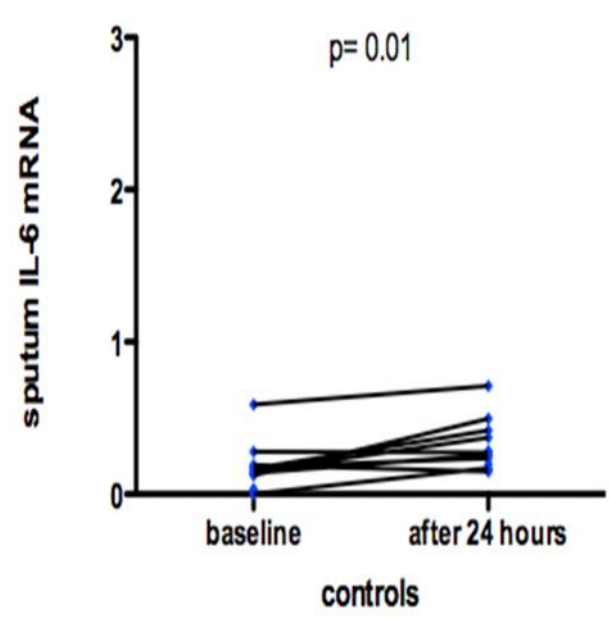


Figure E5a.

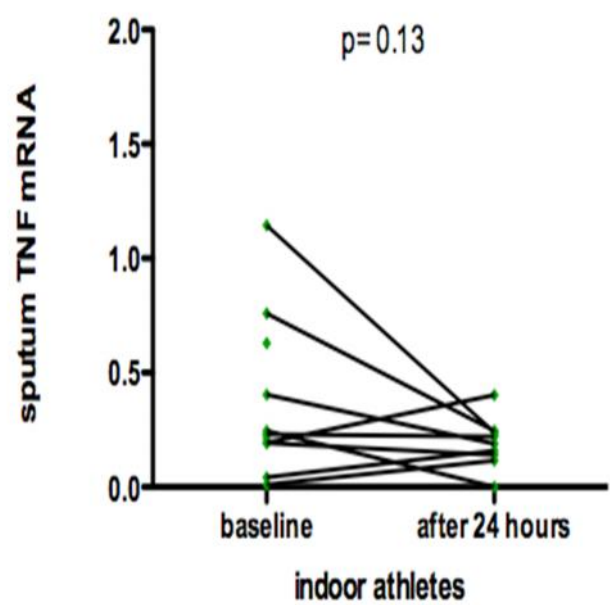


Figure E5b.

