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Original research

Activation of epithelial and inflammatory pathways in adolescent elite athletes exposed to intense exercise and air pollution

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ABSTRACT

Rationale Participation in high-intensity exercise in early life might act as stressor to the airway barrier.

Objectives To investigate the effect of intense exercise and associated exposure to air pollution on the airway barrier in adolescent elite athletes compared with healthy controls and to study exercise-induced bronchoconstriction (EIB) in this population.

Methods Early-career elite athletes attending 'Flemish-Elite-Sports-Schools' (12–18 years) of 4 different sport disciplines (n=90) and control subjects (n=25) were recruited. Presence of EIB was tested by the eucapnic voluntary hyperventilation (EVH) test. Markers at mRNA and protein level; RNA-sequencing; carbon load in airway macrophages were studied on induced sputum samples.

Results 444 genes were differentially expressed in sputum from athletes compared with controls, which were related to inflammation and epithelial cell damage and sputum samples of athletes contained significantly more carbon loaded airway macrophages compared with controls (24%, 95% CI 20% to 36%, p<0.0004). Athletes had significantly higher substance P (13.3 pg/mL, 95% CI 2.0 to 19.2) and calprotectin (1237 ng/mL, 95% CI 531 to 2490) levels as well as IL-6, IL-8 and TNF-α mRNA levels compared with controls (p<0.05). The incidence of EIB in athletes was 9%. The maximal fall in forced expiratory volume in 1 s (%) after EVH test in athletes was significantly associated with prior PM_{10} and $PM_{2.5}$ exposure.

Conclusion Early-career elite athletes showed increased markers of air pollution exposure, epithelial damage and airway inflammation compared with controls. Acute exposure to increased air pollution PM_{10} levels was linked to increased airway hyper-reactivity.

Trial registration number NCT03587675.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Exercise-induced bronchoconstriction (EIB) is a prevalent condition in elite athletes, associated to the type of sport and the environment of exercise.

WHAT THIS STUDY ADDS

⇒ Sputum analysis of adolescent elite athletes showed increased carbon load in airway macrophages, levels of epithelial damage and airway inflammation compared with healthy controls. Furthermore, the airway response to eucapnic voluntary hyperventilation (EVH) testing was associated with prior particulate matter (PM) exposure in these athletes. Finally, underlying mechanisms of EIB are suggested.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The results of our study highlight the association of exposure to air pollution and airway hyper-reactivity in adolescent elite athletes compared with healthy control subjects. Our study is further evidence that action should be taken to reduce PM and improve air quality during training sessions and sports competitions.

Linked

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INTRODUCTION

Although regular physical activity is of utmost importance to prevent worsening of asthma and other chronic diseases,[1] excessive physical activity may induce a stress reaction in the airways resulting in epithelial damage and inflammation.[2] It is known that exercise itself can induce bronchoconstriction in otherwise healthy subjects. This phenomenon is called exercise-induced bronchoconstriction (EIB) and is defined as a transient, reversible airway narrowing occurring during or after exercise.[3] Elite athletes have a high risk to develop EIB, ranging from 30% to 70% of the athletes.[4] Endurance sports and sports in combination to environmental factors such as chlorine or cold air are linked to the appearance of EIB.[5 6] Indeed, the International Olympic Committee (IOC) systematic review and meta-analysis found the highest prevalence of lower airway dysfunction in endurance (25.1%), aquatic (39.9%) and winter (29.5%) athletes.[7] Several mechanisms contributing to EIB have been described: airway cooling-rewarming, airway dehydration, epithelial cell damage resulting in the release of different inflammatory mediators and neurogenic inflammation.[8] Worldwide, different

school programmes exist, with the aim of selecting and training future elite athletes already at a young age. Furthermore, it has been demonstrated that EIB can already be present early on in the sports career or during adolescence.[9 10]

The relationship between air pollution, EIB and elite sports has not been extensively studied. Nowadays, in high traffic areas, air pollution is a major issue and the negative effects of pollutants on the airways have been shown repeatedly.[11 12] It appears to be related to increased oxidative stress and inflammation in airways as well as systemic inflammation, even compromising sports performance.[13 14] During exercise, ventilation increases up to 150 L/min in healthy adults and even beyond 200 L/min in elite athletes, resulting in an elevated inhalation and exposure of potential harmful environmental triggers.[4 15 16] The term air pollution includes particulate matter (PM) and gaseous compounds like ozone (O_3).[17] PM is categorised based on particle seize: PM_{10} (<10 µm), $PM_{2.5}$ (<2.5 µm) and ultrafine particles (<0.1 µm). Daigle *et al* already demonstrated increased ultrafine carbon deposition during exercise compared with rest,[18] and McDonnell *et al* demonstrated a decrease in forced expiratory volume in 1 s (FEV_1) after exercise in exposure of O_3 compared with the same exercise performed in a filtered air environment.[19]

Sputum induction is an important non-invasive tool of airway sampling.[20] Furthermore, transcriptomic analysis on these sputum samples by next-generation sequencing (RNA-Seq) allows high-throughput and detailed characterisation of gene expression profiles.[21] In this way, we wanted to study the inflammatory response to exercise and associated environmental exposures (in particular air pollution) in early-career athletes, since the potential of repetitive bouts of high intensity exercise may lead to chronic inflammation, compared with healthy controls. We hypothesise that the increased ventilation rate of adolescent elite athletes, in a strongly polluted area as Belgium, is associated with airway hyper-reactivity and is reflected in their transcriptomic pattern.

METHODS

Subjects

Early-career elite athletes of the four most prevalent sport disciplines attending 'Flemish Elite Sports' Schools' (12–18 years) were recruited. An overview of the number of athletes per sport discipline in each Elite Sports' Schools is presented in figure 1A. Athletes were included in this study from January 2019 to December 2019. Control subjects, performing less than 6 hours of sport/week, were recruited to and included from January 2020 to March 2021 (figure 1B). Athletes received one visit at their Elite Sports' Schools. The study visit of control subjects was performed in UZ Leuven.

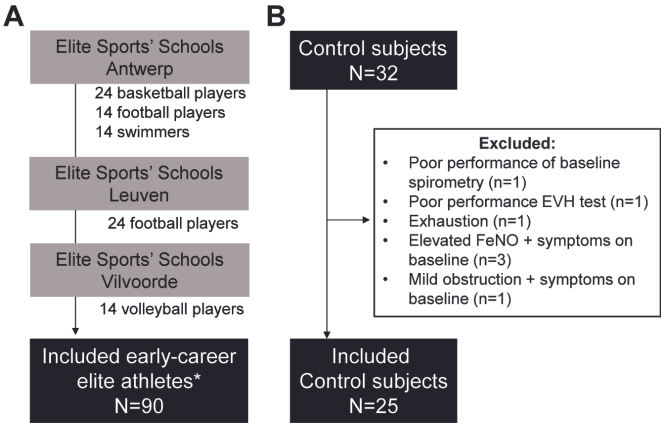

Figure 1 Recruited subjects. (A) Included athletes (n=90) in elite Sports' Schools in Antwerp, Leuven and Vilvoorde. *None were excluded. (B) Included controls (n=25). Exclusion criteria are shown.

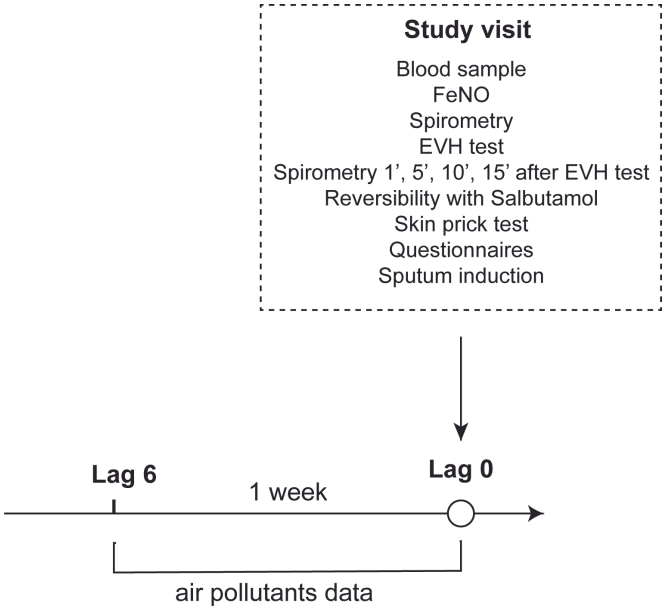

Figure 2 Study design. Demonstrating order of different interventions at the study visit. EVH, eucapnic voluntary hyperventilation.

Study design

The study design is presented in figure 2.

FeNO

Fractional exhaled nitric oxide (FeNO) levels were measured with portable device 'Niox Vero' (Accuramed, Belgium), recorded in parts per billion (ppb).

Spirometry and eucapnic voluntary hyperventilation test

Spirometry was performed using a portable spirometer (Spirolab III Spirometer, MIR). The eucapnic voluntary hyperventilation (EVH) test (EucapSys, SMTEC, Switzerland) was performed according to American Thoracic Society (ATS) guidelines and adapted for this young age group.[10 22] Briefly, subjects inhaled a dry air mixture containing 5% CO_2 at room temperature for 6 min. The target ventilation to be maintained was $21 \times FEV_1$, equivalent to 70% of MVV. The EVH test was considered to be positive if the fall in FEV ≥10% at one of the time points (1', 5', 10', 15') after the EVH test (the measurement after 1' not taken into account). After the EVH test, reversibility testing was done using 400 µg of salbutamol.

Skin prick tests

A skin prick test for nine common aero-allergens was performed on all subjects: grass pollen, mixed tree pollen (hazel, birch and alder), birch pollen, weed pollen, house dust mite (*Dermatophagoides pteronyssinus*), cat, dog, *Alternaria Alternata*, *Aspergillus fumigatus* (HAL Allergy, Leiden, The Netherlands). A subject was considered to be atopic if at least one allergen was positive (≥3 mm and at least the size of the histamine control).[23]

Questionnaires

The Allergy Questionnaire for Athletes (AQUA),[24 25] as well as the asthma control test,[26] was filled in by all subjects. Additionally, a self-made exposure questionnaire, based on previous cohort studies,[10 27] was filled in to assess presence of airway symptoms (eg, wheezing, dyspnoea, coughing, rhinorrhoea, …), medication use, family history of allergies and exposure at home (pets, smoking) and hours of sports a week.

Sputum induction

Subjects inhaled salt solutions of respectively 3%, 4% and 5% during 7 min each. After inhalation, sputum was collected by spitting in a collection tube and processed by the selected plug method, to minimise contamination with saliva, like previously described.[27–31] Sputum induction was not performed in swimmers, due to lack of consent in that particular sport branch. Cytospins (Shandon cytocentrifuge) were prepared from 12 500 and 25 000 sputum cells for differential cell counts and were stained with Diff-Quik. The remaining cells were lysed for mRNA analysis at −80°C. The sputum supernatants were stored at −80°C for further analysis.

Sample analysis

Carbon load in airway macrophages

Black carbon (BC) load in airway macrophages was determined as previously described by Bai *et al*.[32] Briefly, digital images of 25 randomly selected airway macrophages from each cytospin slide were obtained at ×1000 magnification. Cells were manually delineated and the ImageJ software (NIH, Maryland, USA) automatically counted the number of particles and percentage area occupied by BC in the indicated area. The percentage of loaded macrophages was determined manually.

RNA isolation, cDNA synthesis, qPCR

Sputum levels of chemokines (CCL3, IL-8), cytokines (IL-1α, IL-1β, IL-6, IL-17A, IL-17F, IFN-γ, TNF-α), tight junctions (CLDN1, CLDN15, OCLN, ZO-1) and enzymes (CHIT1) were measured using real time qPCR.[27–31] More information can be found in online supplemental material and methods.

RNA-Seq

Sequencing of mRNA was performed on, respectively, the Illumina 4000 (Illumnia, San Diego, USA). A detailed description on library preparation, bioinformatics processing and differential gene expression analysis is available in online supplemental data.

Serum and sputum supernatant biomarker analysis

Serum clara cell protein 16 (CC16) and uric acid levels; and sputum supernatant uric acid, surfactant protein D (SpD), human high mobility group protein B1 (HMGB1) and substance P were measured by ELISA. More information can be found in online supplemental material and methods.

Environmental exposure data

The average air pollution data, more specifically $PM_{2.5}$, PM_{10}, BC and O_3 were obtained from 'Belgian Interregional Environment Agency' (IRCEL). Average regional levels of these marker for each athlete's Elite Sports' School/boarding school address were estimated using a spatial temporal interpolation method. For control subjects, the residence address was used. This model

Table 1 Subject characteristics

				Sport disciplines			
	Control subjects	Athletes	P value	Basketball players	Football players	Volleyball players	Swimmers
No (n=)	25	90		24	38	14	14
Age (years)	15.59±1.64	15.53±1.41	0.9225	15.88±1.30	15.39±1.22	16.64±1.08	14.21±1.31
Gender (M/F)	13/12	51/39	0.8204	20/4	18/20	4/10	9/5
BMI	19.84±3.00	20.79±3.06	0.1677	22.35±2.53**	19.53±3.56	21.35±1.65	21.00±2.10
Atopy (n=)	9 (36%)	42 (47%)	0.3423	10 (42%)	14 (37%)	7 (50%)	2 (14%)
FEV_1 (L)	3.83±0.77	4.11±0.88	0.1391	4.78±0.86**	3.57±0.66	4.29±0.50	4.25±0.89
FEV_1% predicted	104.4±9.5	108.0±12.4	0.1890	104.6±10.3	105.0±11.8	107.6±11.7	121.1±10.7***
FVC (L)	4.35±0.89	4.81±1.09	0.0569	5.58±1.08**	4.12±0.81	5.00±0.50	5.17±1.24
FVC% predicted	103.5±10.0	109.6±14.1	0.0447	105.1±12.5	106.1±11.9	109.7±11.1	126.6±13.0****
FeNO (ppb)	10.0 (7.5–14.5)	14.0 (10.0–23.0)	0.0114	18.5 (13.3–39.0)*	12.5 (9.0–22.3)	13.0 (10.0–18.0)	13.5 (9.8–22.5)
Achieved target ventilation (%)	97.2±15.6	94.7±16.3	0.4924	95.9±18.8	94.3±13.8	94.0±17.8	92.4±12.6
Training years	/	10 (9–11)	/	10 (9–11)	10 (9–12)	10 (7–11)	10 (9–11)
Hours of sports a week	4 (3–5)	20 (16–22)	<0.0001	20 (18–22)****	18 (15–20)****	20 (20–22)****	21 (16–22)****
Smoking state (yes/no)	3/22	1/89	0.0318	0/24	1/37	0/14	0/14
Sputum total cell count (×10^6)	0.4 (0.3–0.9)	1.0 (0.5–1.8)	0.0341	1.2 (0.5–4.7)	0.9 (0.4–1.5)	0.9 (0.6–2.0)	NA
Sputum macrophages (%)	98.0 (92.3–98.8)	96.4 (89.2–99.1)	0.4288	94.0 (59.0–100.0)	97.7 (91.4–99.25)	94.1 (93.6–98.3)	NA
Sputum neutrophils (%)	1.0 (0–5.6)	1.2 (0–9.8)	0.9382	1.0 (0–40.0)	1.2 (0––6.6)	2.8 (0–6.2)	NA
Sputum eosinophils (%)	0 (0–0)	0 (0–0)	0.8758	0 (0–0)	0 (0–0)	0 (0–0)	NA
Sputum lymphocytes (%)	0.8 (0.3–1.2)	0.6 (0–2.0)	0.9068	0 (0–1.7)	1.3 (0.4–2.5)	0.6 (0–1.4)	NA

P value represents the p value obtained with statistical analysis among controls and whole athletes' group. Normally distributed data are represented as mean±SD and analysed viat-test. Non-parametric data are represented as median with IQR and analysed with Mann-Whitney U test.
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared with control subjects (one-way ANOVA for parametric data and Kruskall-Wallis test for non-parametric data).
ANOVA, analysis of variance; BMI, body mass index; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; NA, not available.

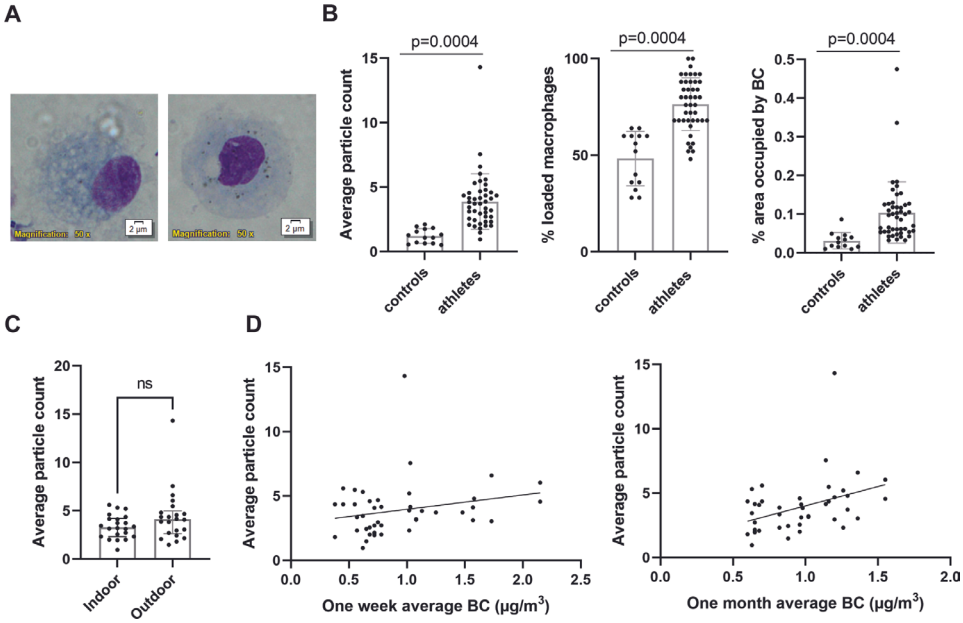

Figure 3 Increased carbon load in airway macrophages in athletes compared with controls. (A) Illustration of images captured for analysis showing airway macrophages stained by diff-Quick with increasing BC load. (B) The average particle count per macrophage, percentage of loaded macrophages and the percentage area occupied by BC for each participant was calculated by a blinded researcher. For each participant, 25 macrophages were counted. (Normality confirmed, unpaired t-test with Welch's correction), controls (controls: n=14, athletes n=44) (C) Comparison of indoor (n=22) and outdoor (n=22) average BC load (normality confirmed, unpaired t-test). (D) Correlation between one week and one month average BC (µg/m^3) and the average particle count observed in athletes (n=44). (Spearman correlation). BC, black carbon; ns, not significant.

provides interpolated daily pollutant values in 4×4 km^2 grids from the Belgian telemetric air quality networks. Individual daily mean concentrations (µg/m^3) were calculated during a 7-day period prior EVH testing. The average outdoor temperature and humidity were obtained from 'Royal Meteorological Institute of Belgium'. Environmental data were used to evaluate the association with response to EVH test. Exposure levels of each pollutant were divided into three subgroups based on data display IRCEL and WHO guidelines (2019): $PM_{2.5}$ low (<10 µg/m^3), immediate (10–25 µg/m^3) and high (>25 µg/m^3); PM_{10} low (<20 µg/m^3), immediate (20–50 µg/m^3) and high (>50 µg/m^3); O_3 low (<50 µg/m^3), immediate (50–100 µg/m^3) and high (>100 µg/m^3).

Statistical analysis

Statistical analysis was performed with Graphpad Prism V.9 (Graphpad Software, San Diego, USA). Normality was tested with Shapiro-Wilk test. To compare the means of two normally distributed groups, parametric t-tests were used, if not normally distributed, the Mann-Whitney test was used. Welch's correction was applied to correct for unequal sample size. Bonferroni was applied to correct for multiple testing. For normally distributed data one-way analysis of variance with Tukey's multiple comparisons test was used to compare a parameter between more than two groups, the Kruskal-Wallis test with Dunn's multiple comparisons test for non-normally distributed data. Contingency tables were tested with Fisher's exact test or χ^2 test. Correlation was studied by the Pearson or Spearman correlation test, depending on normality. IBM SPSS V.28 Statistics (SPSS, Chicago, USA) was used for linear regression analysis. The absolute value of the maximal fall in FEV_1 was log-transformed to obtain homoscedasticity. To investigate the association between the maximal fall in FEV_1 and air pollution exposures, we adjusted the models for age-squared, gender, body mass index (BMI), atopic state, humidity and temperature. Since the dependent variable (maximal fall in FEV_1) was log transformed, the resulting regression coefficients and their 95% CIs were transformed to [10^(B – 1) × 100]. This transformation allows interpreting the coefficient as the percentage of change in maximal fall in FEV_1. A difference was considered significant when $p<0.05$.

RESULTS

Subject characteristics

Ninety adolescent elite athletes from four different sport disciplines were recruited: basketball (n=24), football (n=38), volleyball (n=14) and swimming (n=14) and 25 healthy controls between the ages of 12 and 18 were included. An overview of the subject characteristics is shown in table 1. All athletes performed sport activities on a high level, with a median training load of 18–21 hours/week. Baseline FEV_1% predicted of all athletes exceeded 80%, except for 1 football player previously diagnosed with asthma by physician having an FEV_1% of 69%. Significantly higher baseline FEV_1% and forced vital capacity (FVC%) predicted values were observed in swimmers (mean±SD: 121%±11%) compared with all other sport disciplines and controls. In addition, 33 of 90 early-career athletes (37%) were atopic, mostly to grass pollen (n=25/33), followed by house dust mite (n=22/33), of which 25 were polysensitised.

Cellular composition of the sputum

In total 73 sputum samples of athletes (81%) (swimmers were not included due to lack of consent) and 18 sputum samples of controls (72%) were collected (n=91). Of all samples, 65% yielded a sufficient quality to perform differential cell count (n=59) to phenotype airway inflammation (table 1, online supplemental figure E1). The proportion of cell types (and overall total yields) did not differ between athletes and controls.

The median percentage of squamous epithelial cells on the total cell counts was 16.7% (P25–P75: 6.6%–30.6%). The differential cell count of the sputum samples from these subjects was dominated by macrophages (median: 97%, P25–P75: 91%–99%), followed by neutrophils (median: 1%, P25–P75: 0%–8%).

BC particles in airway macrophages

To assess personal exposure to combustion derived particles, carbon loading in airway macrophages was determined. Of the in total 91 collected sputum samples, 58 samples (64%) yielded a sufficient quality and number of airway macrophages (figure 3A and online supplemental figure E1). There was no association between the carbon content of airway macrophages and age, weight, height or BMI of the subjects. Sputum samples of athletes contained significantly more loaded airway macrophages compared with controls ($p<0.0004$) (figure 3B). Similarly, the number of particles in macrophages and percentage area occupied by BC were significantly increased in athletes compared with controls ($p<0.0004$) (figure 3B). However, no significant difference was observed between indoor and outdoor athletes (figure 3C). One week and the past one month average BC exposure did both not differ between controls and athletes (online supplemental figure E2). The carbon load in macrophages was positively associated with the estimated past one-month average BC ($r=0.3996$, $p=0.0072$) rather than with the shorter time period of one-week average BC in athletes ($r=0.1570$, $p=0.3087$) (figure 3D).

Airway inflammation in controls and athletes

To assess the transcriptomic difference between airway cells of athletes and controls in depth, RNA-Seq was performed on isolated RNA from 48 sputum samples (selected based on quality requirements, online supplemental figure E1). A total of 444 genes were differentially expressed between controls ($n=11$) and athletes ($n=37$) ($p<0.05$). Seventy-seven genes were upregulated and 367 genes were downregulated in athletes compared with controls. DEGs were mapped onto a volcano plot (figure 4A, online supplemental table E2). Specifically, DEGs related to inflammation and epithelial cell damage including TNF, CCL3, CLDN15, TNAIP3, IL17RC and TLR3, respectively, were observed. To identify related mechanisms, DEGs were subjected to Ingenuity Pathways Analysis (IPA) analysis. Significant canonical pathway analysis revealed that those DEGs play key roles in gene networks involved in cell death and survival and immune cell trafficking (figure 4B). Similarly, gene set enrichment analysis demonstrated that gene set of airway inflammation, including TNF-α, INF-γ and IL-6 were enriched for athletes (figure 4C).

After enrichment analysis, several key genes were identified and validated using qPCR (controls: $n=6$, athletes: $n=43$, online supplemental figure E3). We observed significantly elevated transcription levels of TNF-α and CCL3 in sputum of athletes compared with controls even correlating with the number of sports/weeks. CLDN15 was not significantly decreased in athletes compared with controls, but was significantly different among the different sport disciplines ($p=0.0135$), with the highest levels observed in volleyball players. Besides TNF-α, athletes had significantly higher levels of sputum IL-6 and IL-8 mRNA levels compared with controls (figure 4D, $p<0.05$). Furthermore, IL-6 levels were significantly higher in outdoor athletes compared with indoor athletes ($p=0.0049$). Other measured cytokines (IL-1α, IL-1β, IL-17A, IL-17F, IFN-γ) did not show a significant difference between controls and athletes (online supplemental figure E4).

Damage-associated molecular pattern, including HMGB-1 and SpD, was not detectable in sputum from controls, while present in sputum of athletes ($n=24$, respectively) (data not shown). Serum CC16 levels, described as marker of epithelial damage in serum from athletes in previous cohorts, were surprisingly not significantly elevated in athletes compared with controls (figure 4E) and also did not correlate with the maximal fall in FEV_1 after EVH test. A significant higher calprotectin level in sputum supernatant, a marker for neutrophilic inflammation, was however observed in athletes when compared with controls (figure 4F, $p=0.0002$). Sputum neutrophil levels furthermore correlated positively with calprotectin levels in sputum supernatant ($r=0.3216$, $p=0.0312$). In addition, athletes demonstrated increased levels of substance P in sputum supernatant compared with controls (figure 4G, $p=0.0103$).

EVH response in young elite athletes

Eight elite athletes (9%) tested positive for EIB (≥10% fall in FEV_1) (online supplemental table E7). Because of clinical symptoms during the test (dyspnoea), one additional athlete did not achieve three post-EVH spirometry measurements and salbutamol (Ventolin) was given early after symptom occurrence and hence she was excluded from analysis. Of the EIB+ athletes ($n=8$) three were atopic (online supplemental figure E5). Of these atopic EIB+ athletes, two athletes were basketball players (2/24) and one was a volleyball player (1/14). In total three of the non-atopic EIB positive athletes were swimmers (3/14) and two were football players (2/38). Also, three control subjects (12%) tested positive for EIB of which two were atopic. The EIB response was mild in seven athletes and three controls, that is, 10%–25% fall in FEV_1 and was considered to be moderate (≥25% to <50%) in one athlete. Nine subjects had their maximal fall in FEV_1 (%) under threshold of 10% within 10 min after the EVH test and two controls had a decline in FEV_1 later on, 15 min after the EVH test. Preliminary comparison between atopic EIB+ ($n=2$) and EIB− ($n=4$) athletes by RNA-Seq can be found in online supplemental file (please refer to figure E5 and table E4).

Air pollution, epithelial barrier integrity and severity of bronchoconstriction during EVH test

The association of environmental exposures such as prior air pollution exposure, outdoor temperature and outdoor humidity with expression of tight junctional proteins was evaluated in all athletes. Figure 5 shows the temporal analysis of pollutant levels during the period of inclusions. Significantly lower mRNA levels of OCLN and ZO-1 in athletes exposed to higher levels of PM_{10} (>20 µg/m^3, $n=22$) compared with athletes exposed to lower levels (<20 µg/m^3, $n=23$) are observed ($p=0.0284$, $p=0.0286$) (figure 5D). The same trend is observed for CLDN1 mRNA levels ($p=0.0579$) (figure 5D).

Table 2 shows the associations between daily air pollution levels and changes in maximal fall in FEV_1 after EVH test, adjusted for humidity, temperature, age-squared, gender, BMI and atopic state in a single pollutant model. The spearman correlation between meteorological factors and other pollutants are presented in online supplemental table E5. The maximal fall in FEV_1 was significantly associated with both $PM_{2.5}$ and PM_{10}. For example, the maximal fall in FEV_1 was 10.45% lower for each 1 µg/m^3 increment in average $PM_{2.5}$ and 10.42% lower for each 1 µg/m^3 increase in $PM_{10,}$ indicating a reduction in maximal

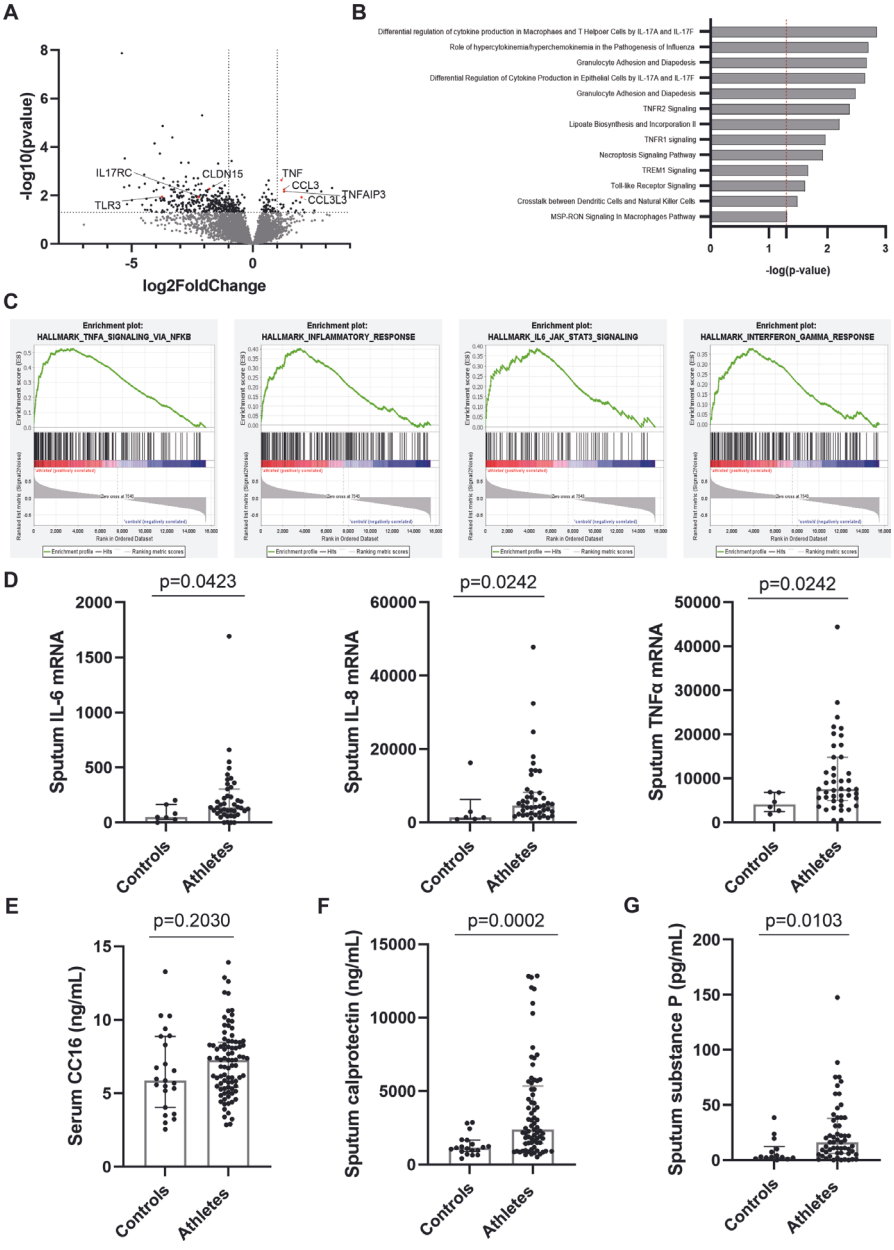

Figure 4 Profiling of serum and induced sputum samples in controls and athletes. (A) Volcano plot with the magnitude expressed as log2 fold change (x-axis) and significance expressed as −log10 of the adjusted p value (y-axis) of differential expression analysis. Genes of interest are labelled. (B) Selected significantly enriched or downregulated pathways based on Ingenuity Pathways Analysis (IPA) analysis listed according their p value. (C) Significant enrichment plots at False Discovery Rate (FDR) <25%. (D) Following cytokine mRNA levels (controls: n=6, athletes: n=43) were measured via qPCR: IL-6, IL-8 and TNF-α. (E) Serum CC16 levels in controls vs athletes were measured via ELISA (controls: n=23, athletes: n=83). Sputum supernatants (controls: n=18, athletes: n=73) calprotectin (F) and substance P levels (G) were measured via ELISA. (Mann-Whitney U test).

fall in FEV_1 with increasing air pollution exposure. Concretely, for an athlete with a maximal fall in FEV_1 of −5%, the maximal fall will decrease to −5.5% when exposed to one unit more of PM. In the multipollutant model, considering both $PM_{2.5}$ and PM_{10}, the association with PM_{10} appeared to be the most robust (table 3).

DISCUSSION

Our results clearly indicate that in the airways of early-career elite athletes, who are exposed to intense physical exercise and air pollution, the epithelial barrier is affected and airway inflammation occurs. Increased carbon load in sputum macrophages of these athletes is observed, whether or not performing outdoor sport activities. Probably, it is the combination of environmental triggers (both intense exercise and air pollution) which impacts the airways. Remarkably, the airway response to EVH testing in athletes was associated with prior PM exposure, suggesting that acute air pollution could induce increased bronchial reactivity of the airways, which is particularly relevant in athletes with high ventilatory demands. In athletes' sputum samples, genes related to epithelial cell damage, airway inflammation (IL-6, IFN-γ, TNF-α) and immune trafficking are clearly upregulated compared with control subjects. Moreover, based on preliminary RNA-Seq analysis between EIB+ and EIB− athletes, the impact of tight/gap epithelial damage, oxidative stress and (neuro)inflammation can be envisioned in research on mechanisms of EIB.

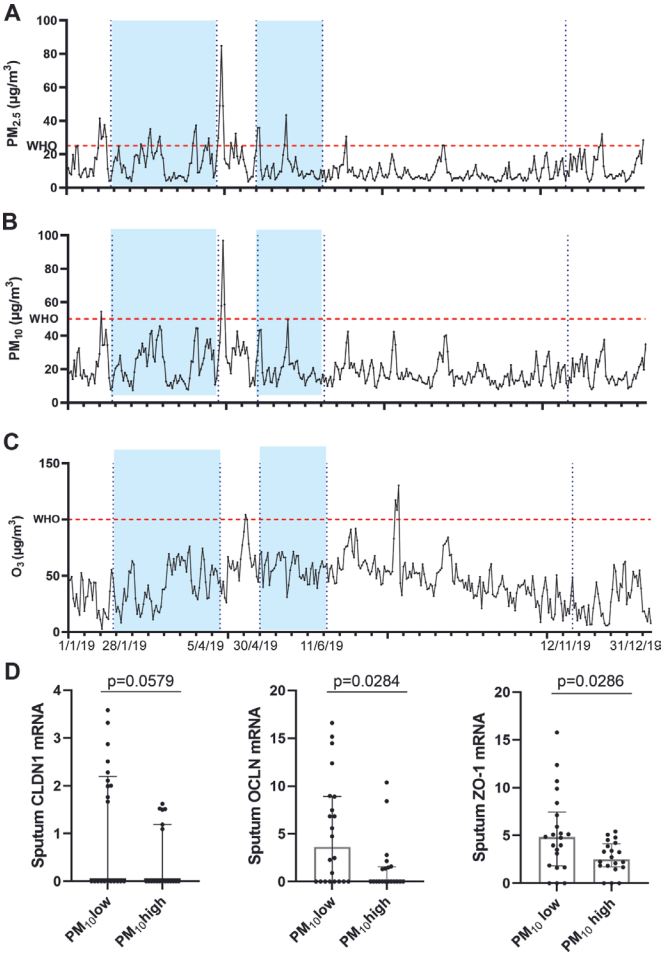

Figure 5 Air pollution exposure prior EVH test. (A–C) Daily mean levels of each pollutant considered during the study period averaged over the three included Elite Sport's Schools. The flashing red line refers to the maximum concentration of each pollutant established by WHO.[57] The dotted blue lines refer to the time frame the athletes participated in the study. (G) Effect of the most robust air pollutant PM_{10} on tight junction mRNA expression of CLDN1, OCLN and ZO-1. (Mann-Whitney) (PM_{10}low: n=22, PM_{10}high: n=20, outliers were removed based on Grubbs). EVH, eucapnic voluntary hyperventilation. O_3, ozone; $PM_{2.5}$, particulate matter < 2.5 µm; PM_{10}, particulate matter < 10 µm.

It is known that acute and/or chronic exposure to intense physical exercise can induce airway inflammatory reactions including cytokine release.[33 34] Indeed, many studies have consistently shown that local and/or systemic levels of IL-1α, IL-1β, IL-6, IL-8 and IL-10 are increased in adult athletes after exercise.[27 35] However, few studies focus on local inflammatory markers in early-career athletes without asthma.[36] We here confirm increased sputum mRNA levels of IL-6, IL-8 and TNF-α in early-career elite athletes. TNF-α can be released from activated macrophages and is able to induce cytokine release from the epithelium. The epithelial barrier might furthermore be directly impacted by intense physical exercise. Epithelial damage was suggested in earlier studies,[10 27 37 38] and we here also observed elevated damage-associated molecular pattern (HMGB-1) in the sputum of the athletes compared with controls, which may feature as early inducers of local inflammation. Furthermore, the impact of the epithelial barrier is reflected by the down-regulation of CLDN15, a component of the tight junctions, in athletes compared with controls. We observed increased sputum neutrophilia in athletes related to the hours of physical training, although no pathological neutrophilic inflammation (defined as sputum neutrophil count >63%) was observed. However, also sputum calprotectin levels were significantly increased in athletes compared with controls, pointing to the potential start of neutrophilic airway inflammation in young elite athletes. In line with this, Decaesteker *et al*[39] demonstrated a significant increase in sputum supernatant calprotectin levels after exposure to exercise, as well as to other environmental conditions (hypoxia and cold).

Furthermore, histological analysis revealed clear uptake of BC particles by the macrophages, confirming their activity, which was increased in athletes compared with controls, likely the result of their high ventilatory demands during exercise. Chronic PM exposure has been linked to higher airway macrophage BC load.[32 40–42] Although the exact lifespan of human macrophages is unknown, $PM_{2.5}$ 6-month exposures are most strongly associated with airway macrophage BC content, rather than with shorter time periods considered.[40 43] We found a significant correlation between airway macrophages BC load and athletes' average BC exposure during 1 month in average, but not for a shorter period of 1 week. Besides, $PM_{2.5}$ and PM_{10} might both impact the airway epithelial barrier. We found significant lower levels of OCLN and ZO-1 levels in athletes exposed to higher levels of PM_{10} compared with athletes exposed to lower levels.

Strikingly, the maximal fall in FEV_1 post-EVH test in athletes was significantly associated with prior PM exposure (at lag3). Previous studies also demonstrated a significant negative correlation between spirometric indices (FEV_1 and FVC) and the exposure to air pollution 3–6 days prior.[44 45] For the EVH test, such correlation was not described before. However, variability in the results of the EVH test has repeatedly been reported.[46–48] The algorithm for the evaluation of asthma and EIB in athletes by Boulet and O'Byrne in *New England Journal of Medicine* already suggested to repeat the bronchoprovocation in case of a negative result in a period of more intense training or during exposure to relevant allergens or environmental conditions.[49] This environmental condition might very well be PM exposure. In addition, the recent position paper of European Academy of Allergy and Clinical Immunology highlights the importance of repeat assessment and requirement of in season testing.[50]

Table 2 Single pollutant models for $PM_{2.5}$, PM_{10} and O_3

	Adj* B-coefficient	Adj* 95% CI	P value
$PM_{2.5}$	−10.45	−10.21 to −10.72	<0.001
PM_{10}	−10.42	−10.21 to −10.67	<0.001
O_3	−10.07	−9.93 to −10.21	0.289

Adjusted relative changes (%) with their 95% CI in maximal fall in FEV_1.
*Adjusted for humidity, temperature, age-squared, gender, BMI and atopic state.
BMI, body mass index; FEV1, forced expiratory volume in 1 s; O_3, ozone; PM_{10}, particulate matter < 10 µm; $PM_{2.5}$, particulate matter < 2.5 µm.

Table 3 Two-pollutant model with $PM_{2.5}$ and PM_{10}

	Adj* B-coefficient	Adj* 95% CI	P value
$PM_{2.5}$	−9.38	−8.05 to −10.38	0.163
PM_{10}	−11.27	−10.07 to −12.59	0.036

Adjusted relative changes (%) with their 95% CI in maximal fall in FEV_1
*Adjusted for humidity, temperature, age-squared, gender, BMI-squared and atopic state.
BMI, body mass index; FEV1, forced expiratory volume in 1 s; PM_{10}, particulate matter < 10 µm; $PM_{2.5}$, particulate matter < 2.5 µm.

Emphasis thus far was on the risk of airway barrier damage and inflammation by intense physical exercise. However, the potential beneficial advantages were less studied at the gene expression level. To our knowledge, this is the first study that focuses on a more in-depth transcriptomic profiles from human sputum samples of early-career athletes compared with healthy controls by RNA-Seq. This analysis confirmed the role of epithelial damage, immune trafficking and airway inflammation. However, several other genes were found to be differentially expressed between controls and athletes. Of these CCL3 (MIP-1α) was clearly upregulated. CCL3 is known to be involved in neutrophilic inflammation and might be produced by macrophages, lymphocytes, neutrophils, eosinophils, fibroblasts and mast cells.[51 52] IL1-β can induce its expression in airway epithelial cells by activating nuclear factor (NF)-kB. Its expression can be related to our observation of sputum supernatant calprotectin level. Furthermore, the expression profile in our athletes' cohort was significantly associated with the gene set that is upregulated in response to IFN-γ. The rise in INF-γ should be considered as beneficial for immune state, since it is an anti-inflammatory cytokine.[53] In line with previous studies, we found a downregulation of TLR3 in athletes compared with controls.[54 55] This may contribute to the higher reported susceptibility for infections in athletes.[54] However, on a long term, a decrease in TLR might also be beneficial due to reduced inflammatory capacity of leukocytes, limiting chronic inflammation. In addition, we found significantly lower IL17RC, which is a coreceptor to respond to IL-17A and IL-17F.[56] This downregulation might also act as a protective response of athletes against immune inflammation. Taken together our results point towards a type 1 and 17 inflammation on intense exercise in early-career elite athletes.

We are aware of a major limitation of the study that inflammation in swimmers could not be documented due to the absence of sputum samples of swimmers who, as a sport discipline, did not consent to have a sputum induction done. As a result, sufficient qualitive RNA to use for RNA-Seq analysis, was only available from atopic EIB+ athletes, and hence interesting information is missing about the underlying mechanism in non-atopic EIB+ athletes. Another limitation of our study is that the exposure to air pollution was estimated based on environmental measurements in the area where the control subject or athlete resides (either at home or as an intern in the elite sport school) but were not measured by personal samplers, accordingly not taking into account other personal factors such as time spent indoors versus outdoors. In addition, we have no control over the amount of single exposure in each subject. However, this is best resembling the real-life situation, in which athletes are exposed to different triggers at the same moment in time. Lastly, we are aware that our cross-sectional and observational design do not permit causal relations to be drawn. Therefore, mice models are needed.

In conclusion, high intensity exercise and exposure to air pollution in early-career athletes are associated with increased levels of epithelial damage and airway inflammation compared with controls. Acute exposure to increased air pollution PM_{10} levels may be associated with increased airway hyper-reactivity.

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Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by Ethical Committee of KU/UZ Leuven (S59778). Participants gave informed consent to participate in the study before taking part.

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Data availability statement Data are available in a public, open access repository. We will provide the data in open access to allow analysis over different study cohorts in for example a systematic review.

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