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

AUTHORS: Janne Goossens[1]; Anne-Charlotte Jonckheere[1]; Ellen Dilissen[1]; Sven F Seys[1]; Tatjana Decaestecker[1], Camille Goossens[1]; Koen Peers[2]; Vincent Vanbelle[3]; Jeroen Stappers[4]; Sven Aertgeerts[5]; Jasmine Leus[6,7]; Sophie Verelst[7,8]; Marc Raes[7,8]; Lieven Dupont[9,10]; Dominique M Bullens[1,7]

Online Data Supplement

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Supplementary Material and methods

RNA isolation, cDNA synthesis, qPCR

RNA isolation was performed with the Mini RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. Afterwards, RNA concentration and quality were measured with Nanodrop (Thermo Scientific, Whaltham, USA). cDNA was synthesized with the High Capacity cDNA Reverse Transcription Kit (Applied biosystems, Waltham, USA) with adapted protocol for low concentration of RNA. Sputum levels (CCL3, CHIT1, CLDN1, CLDN15, IL1A, IL1B, IL6, IL8, IL17A, IL17F, IFNG, OCLN, TJP1, TNF) were measured using real time qPCR.[1–5] Data was normalized to the geometric mean of the reference genes PPIA and RPL13A, determined with RefFinder.[6] Newly developed primers and probes are listed in supplementary table E1. cDNA plasmid standards were used to quantify the amount of target gene in unknown samples.[7]

RNA-Seq library preparation and sequencing

Additional RNA column purification analysis (Qiagen, Hilden, Germany) was performed on samples to increase the number of samples available for RNA-Seq. RNA libraries were constructed using QuantSeq 3' mRNA library prep kit (Lexogen, Vienna, Austria). A protocol modification for low input/quality RNA was performed on samples with RNA <75-100ng (5µl or 15-20ng/µl) and/or RIN-value <5, according to manufacturer's

guidelines (Lexogen, Vienna, Austria). Samples were indexed to allow for multiplexing. Library quality and size range was assessed using a Bioanalyzer (Agilent Technologies, California, USA) with the DNA 1000 kit (Agilent Technologies, California, USA). Sequencing was performed using the HiSeq 4000 (Illumina, San Diego, USA). Single-end reads of 50 bp length were produced with a minimum of 1M reads per sample.

Bioinformatics processing of RNA-Seq data and differential gene expression analysis

Quality control of raw reads was performed with FastQC v0.11.7, available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc. Adapters were filtered with ea-utils fastq-mcf v1.05[8]. Splice-aware alignment was performed with HiSAT2 against the human reference genome hg38, Ensembl83. Reads mapping to multiple loci in the reference genome were discarded. Resulting BAM files were handled with Samtools v1.5.[9] Quantification of reads per gene was performed with HT-seq Count v2.7.14. Samples containing <700.000 reads were removed for further analysis. Count-based differential expression analysis was done with R-based Bioconductor package DESeq2.[10] Reported p-values were adjusted for multiple testing with the Benjamini-Hochberg procedure, which controls false discovery rate (FDR). Mean normalised counts <5 were removed to correct for low base mean expression.

For canonical pathway analysis the Ingenuity Pathway Analysis (IPA) (Qiagen, Valencia, CA) was used using the gene list of differentially expressed genes (DEGs).

Gene Set Enrichment Analysis (GSEA) was performed with GSEA software (MSigDbv6, Broad Institute) using the full gene lists with normalized reads.

Serum and sputum supernatant biomarker analysis

Serum clara cell protein 16 (CC16) was quantified by ELISA (Biovender, Czech Republic). Serum (1:20 dilution) and sputum supernatant (1:5 dilution) uric acid levels, were determined with the Amplex Red uric acid/uricase assay kit from Invitrogen (Thermo Fisher scientific, Waltham, USA). Surfactant protein D (SpD) levels in undiluted sputum supernatant samples were measured by ELISA (R&D systems, Minneapolis, USA). Human high mobility group protein B1 (HMGB1) levels were analysed in undiluted sputum supernatant samples (Abbexa, Cambridge, UK). Substance P was measured using a human substance P ELISA, with sputum supernatant samples diluted 1:2 (Cayman Chemical, Michigan, USA). Calprotectin levels in sputum supernatant samples (1:20 dilution) were analysed using a calprotectin ELISA kit (CALPRO AS, Lysaker, Norway). All assays were performed according to manufacturers' guidelines.

Goossens J, *et al. Thorax* 2023;0:1–9. doi: 10.1136/thorax-2022-219651

Supplementary results

Upper and lower airway symptoms

Different symptoms related to upper and lower airway disease, both outside and during exercise, were evaluated with the help of a previously reported questionnaire [1,11]. The most reported lower airway symptom during exercise was shortness of breath (Figure E5). Approximately 43% of swimmers (n=6), 36% of volleyball players (n=4), 26% of football players (n=10), 21% of basketball players (n=5) and even 48% of control subjects (n=12) reported shortness of breath during exercise. Wheezing during exercise, which has been described to be associated with a positive EVH test, was most reported in swimmers (22%, n=3). Consistently, swimmers reported higher use of respiratory medication (n=9) (table E6). Similarly, swimmers reported most upper respiratory symptoms during exercise (Figure E6). Six athletes self-reported a positive history of EIB/asthma.

Explorative comparison between EIB+ and EIB- athletes

RNA-Seq analysis of two atopic EIB+ athletes compared to 4 matched atopic EIB- athletes was performed (table E3). (The other sputum samples from EIB+ subjects did not allow further analysis due to quality issues, hence we decided to study atopic subjects only). The PCA plot showed 2 clear separate populations (Figure E5). After correction for multiple testing, 372 genes with an effect size of 0.05 were significantly

Goossens J, *et al. Thorax* 2023;0:1–9. doi: 10.1136/thorax-2022-219651

differentially expressed between EIB+ and EIB- athletes (271 up- and 101 downregulated, Figure 5C, table E4). IPA analysis, identified increased pathways for oxidative signalling and neuroinflammation for EIB+ athletes compared to EIB- athletes (Figure E5). Other pathways associated with the DEGs included interferon signalling, tight junction and gap junction signalling. Enrichment analysis confirmed the role of oxidative and type I IFN-α response in EIB+ athletes (Figure E5).

Effect of air pollution on transcriptome

To investigate the effect of air pollution on the airway transcriptome of athletes, we performed differential expression analysis based on the exposure level of the athletes to each air pollutant; $PM_{2.5}$ <10 µg/m^3 (low) vs >25 µg/m^3 (high), PM_{10} <20 µg/m^3 (low) vs 20-50 µg/m^3 (immediate) (no values above threshold of 50 µg/m^3) and O_3 <50 µg/m^3 (low) vs 50-100 µg/m^3 (immediate) (no values above threshold of 100 µg/m^3), respectively (Figure E7). The training hours a week and training years did not differ amongst the different sport disciplines (table 1). In addition, the transcriptomic profiles did not differ amongst the different sport disciplines (table E8). Athletes exposed to higher levels of $PM_{2.5}$ (n=4), significantly expressed more CHIT1, a marker of activated human macrophages, compared to athletes exposed to low levels of $PM_{2.5}$ (n=12) (Figure E7, E8). The downregulated genes in this group included MUC1, MUC4 and ECM1 (table E9). In contrast, the differential expression analysis based on PM_{10}, low

(n=22) versus immediate (n=23) revealed only 2 differentially expressed genes namely FOLR3 and KRT80 (Figure E7). There were no genes differentially expressed in athletes exposed to low (n=18) compared to immediate O_3 levels (n=27) (Figure E7). In addition, air pollution exposure is known to induce epithelial barrier dysfunction. We found a trend towards elevated CC16 serum levels in the group of athletes exposed to higher levels of $PM_{2.5}$ (n=4) compared to athletes exposed to low levels of $PM_{2.5}$ (n=12) (p=0.0557) (Figure E7). Another DAMP released from ischemic tissues and dying cells, serum uric acid levels, significantly correlated with the concentration O_3 (p=0.0002, r=0.3847) at lag 0 (Figure E7). Also, sputum IL-17F mRNA levels correlated with O_3 concentration at lag 0 (p= 0.0369, r= 0.3231).

Supplementary discussion

Preliminary comparison EIB+ and EIB- athletes

Our RNA-Seq analysis studying differences between EIB- atopic athletes and EIB+ atopic athletes should be considered 'exploratory' due to the low sample numbers available. Nevertheless, it reveals interesting suggestions concerning the underlying mechanisms of EIB pointing to oxidative stress and data will be provided in open access to allow analysis over different cohorts. Oxidative stress induces mitochondrial damage, can activate nuclear factor (NF)-κB, which is known to play a critical role in mediating immune and inflammatory responses and apoptosis.[12] Elite sport activities already have been described as stimulus able to induce oxidative stress,[13] but our study suggests a link with EIB. Further research should furthermore focus on oxidative stress especially in the airways of EIB+ athletes. We here furthermore suggested activation of the neuroinflammation signalling pathway in EIB+ athletes. Neuropeptides such as substance P and neurokinin A are also described by others to be involved in EIB.[14] We found an association of the gene set comprising genes up-regulated in response to IFN-α with EIB+ athletes. Recent studies have demonstrated that IFN-α negatively regulates Th2 function, suggesting a protective role. However, recent viral infections, which may also trigger EIB, may also be responsible for this observed upregulation in EIB+ athletes.

Supplementary figures

Supplementary tables

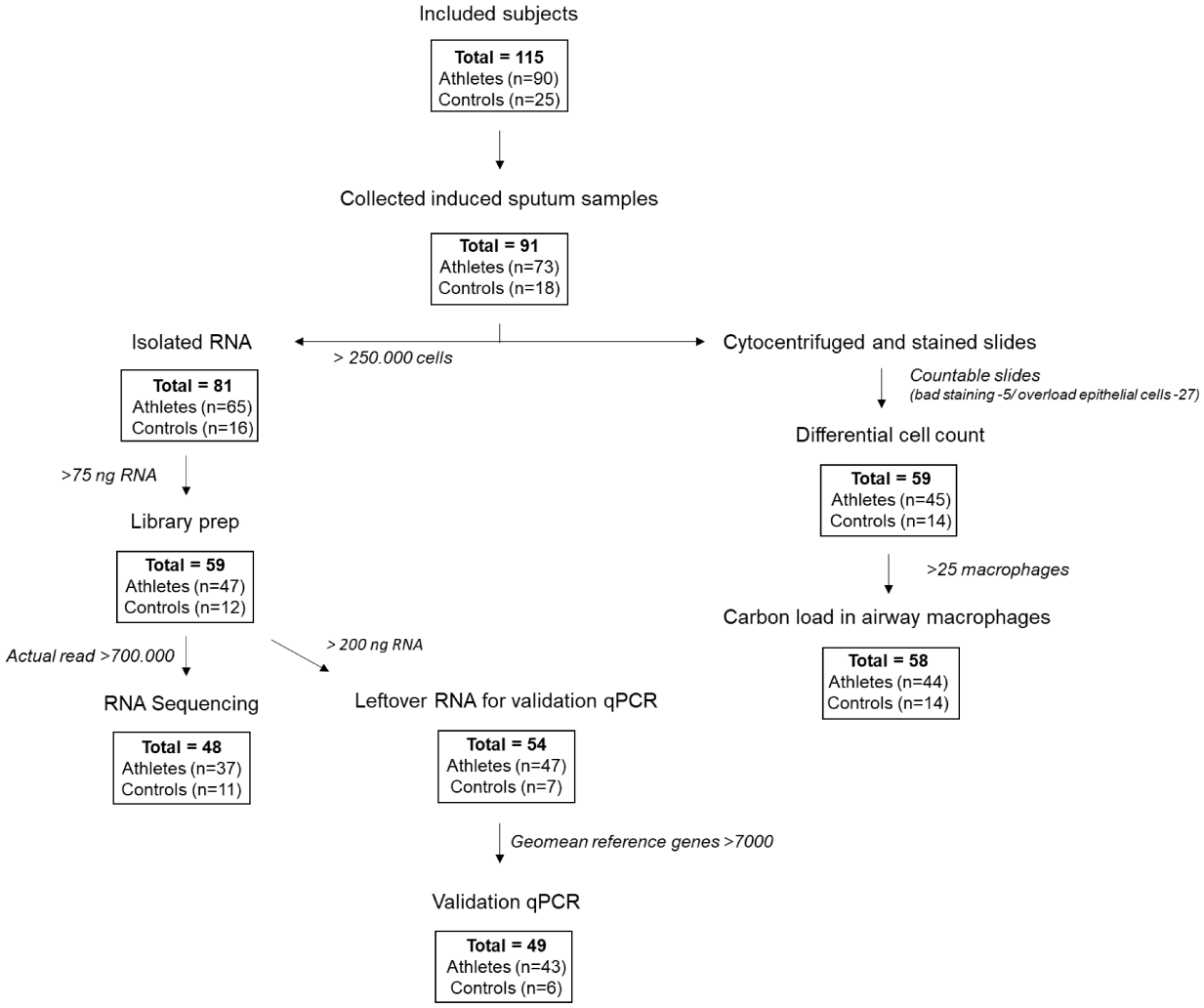

Figure E1. Flow diagram sputum sampling

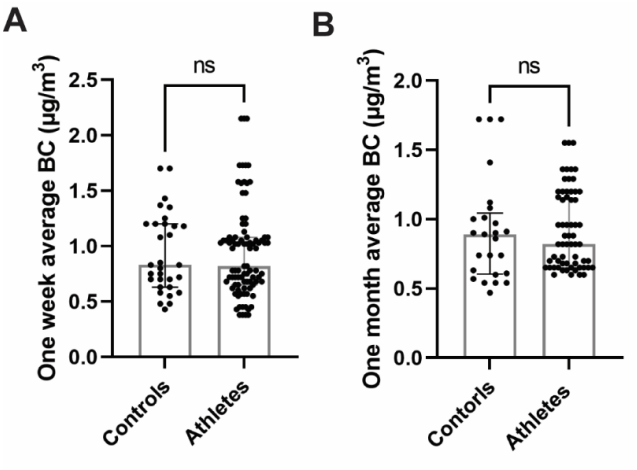

Figure E2: One-week and one-month average BC exposure in controls vs athletes

Comparison of one week (A) and one-month (B) average BC (µg/ m3) between controls and athletes. (Mann-Whitney test)

Goossens J, *et al. Thorax* 2023;0:1–9. doi: 10.1136/thorax-2022-219651

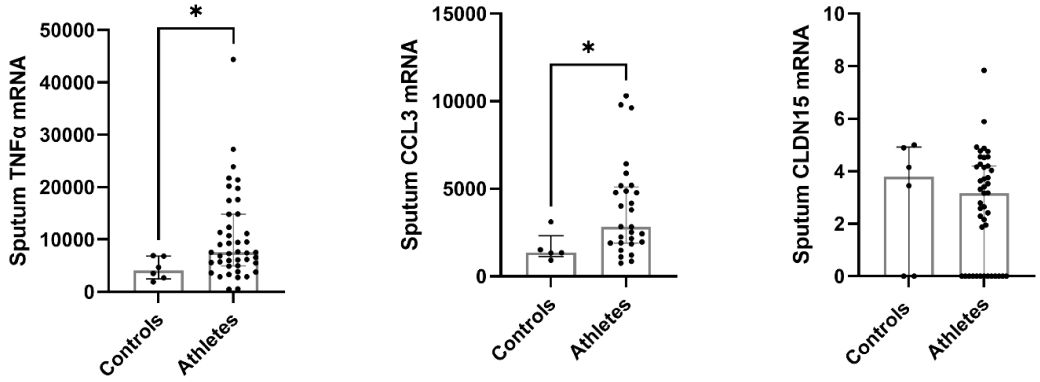

Figure E3: Validation qPCR controls vs athletes

Remaining RNA for controls (n=6) and athletes (n=43) was used to validate obtained results via qPCR. (Mann-Whitney test) *p<0.05.

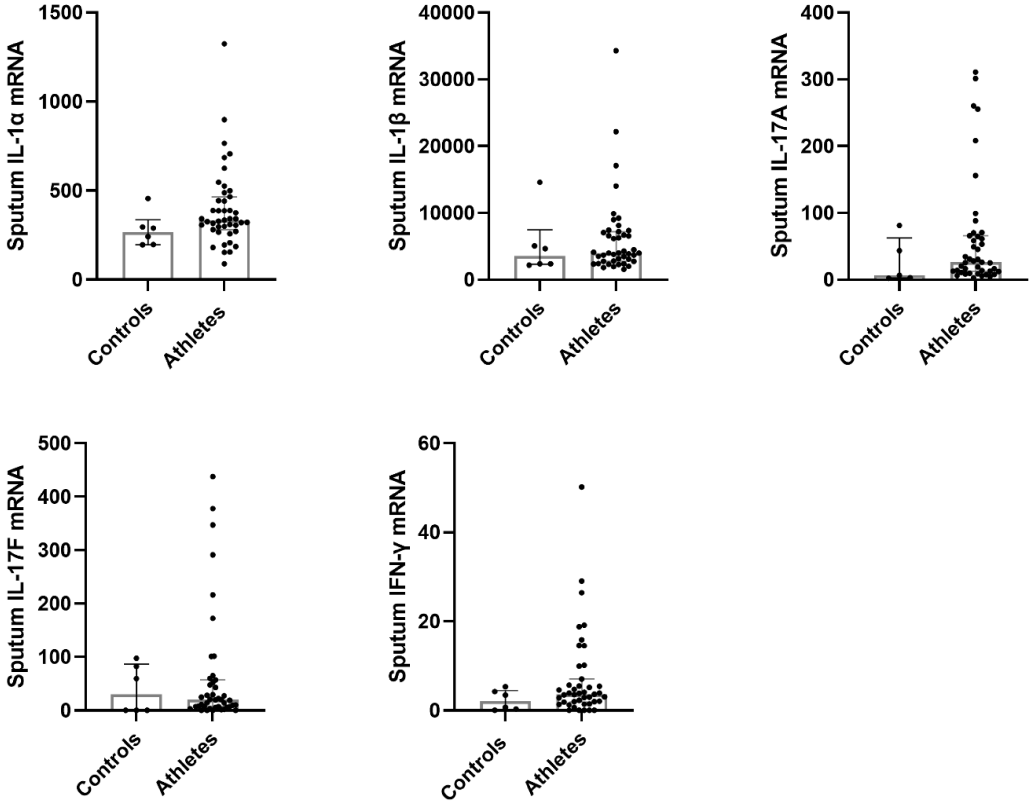

Figure E4: qPCR cytokines controls vs athletes

Remaining RNA for controls (n=6) and athletes (n=43) was used to study the cytokine profile in controls vs athletes via qPCR. (Mann-Whitney test)

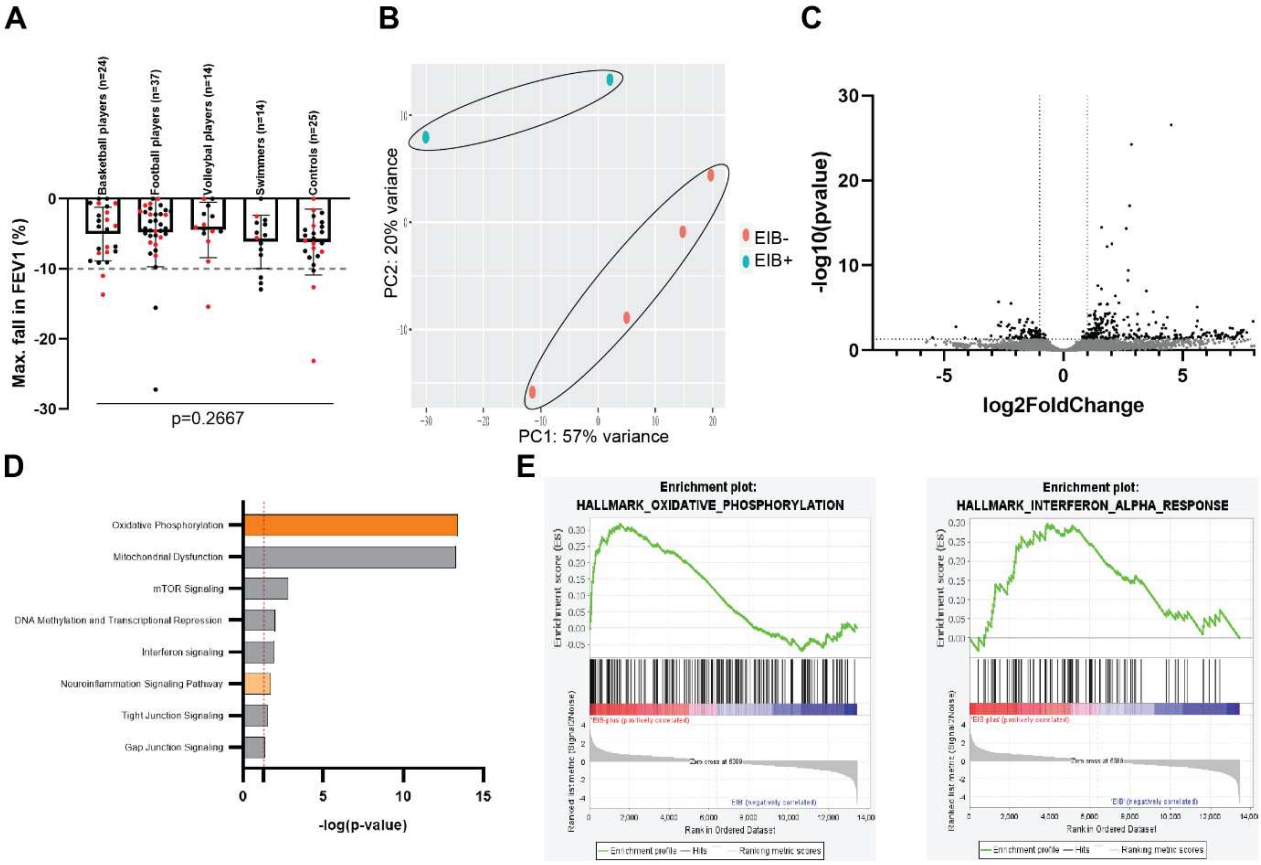

Figure E5: Transcriptome profiling of induced sputum samples in EIB- and EIB+ athletes. (A) EVH test was completely performed in basketball players (n=24), football players (n=37), volleyball players (n=14), swimmers (n=14) and control subjects (n=25). The test was considered positive if a drop in FEV ≥ 10% was observed on at least one time point after the EVH test. Red dots represent atopic subjects. (Kruskal-Wallis test) **(B)** Principal-component analysis (PCA) plots of EIB+ athletes (blue) and EIB- athletes (red). **(C)** 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. **(D)** Selected significantly enriched or downregulated

pathways based on IPA analysis listed according their p value. Orange bars: positive z-score; grey bars: no activity pattern available **(E)** Significant enrichment plots at FDR < 25%.

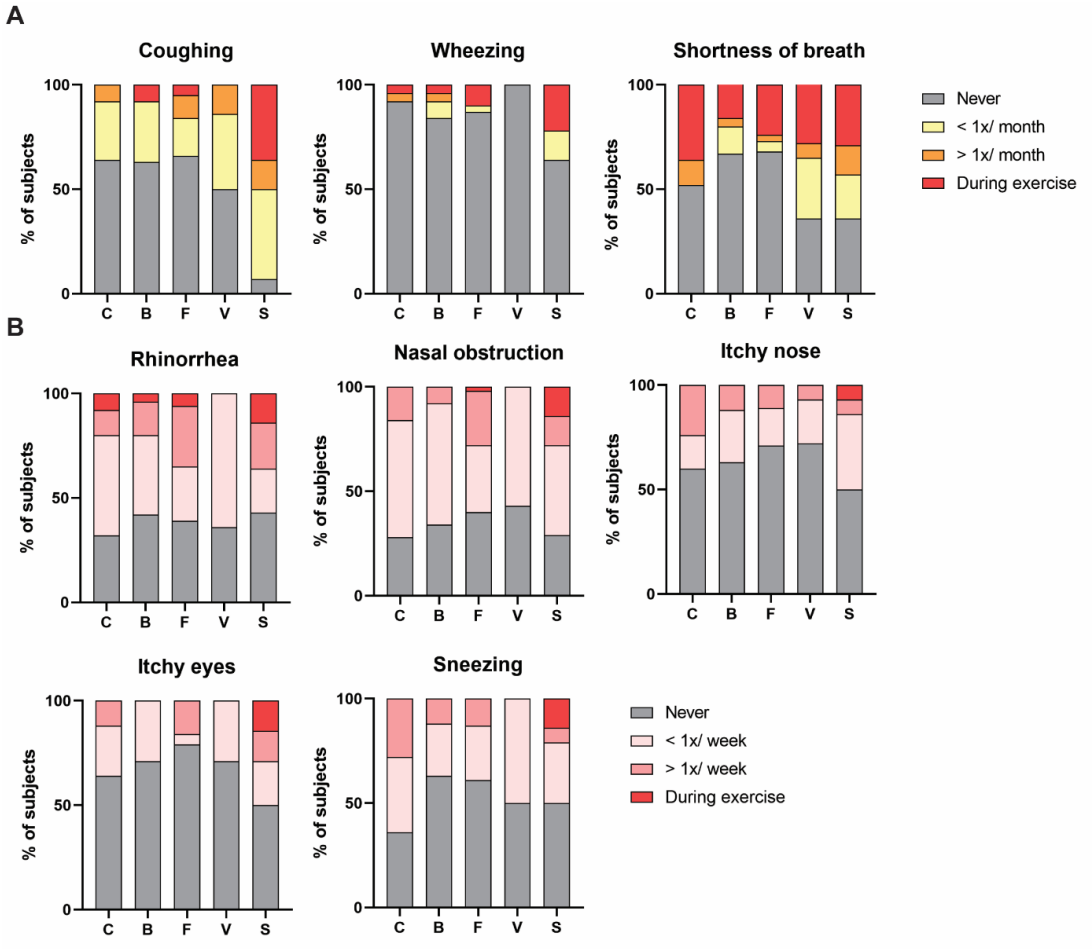

Figure E6: Self-reported upper and lower airway symptoms

Reported symptoms in lower (A) and upper airways (B) were evaluated via a symptom questionnaire in controls ('C', n=25) basketball ('B', n=24), football ('F', n=38), volleyball players ('V', n=14) and swimmers ('S', n=14). Data are expressed as the percentage of subjects reporting a specific symptom during exercise, >1 x/ month (or /week for the upper airways), < 1x/ month (or /week for the upper airways or never).

Goossens J, *et al. Thorax* 2023;0:1–9. doi: 10.1136/thorax-2022-219651

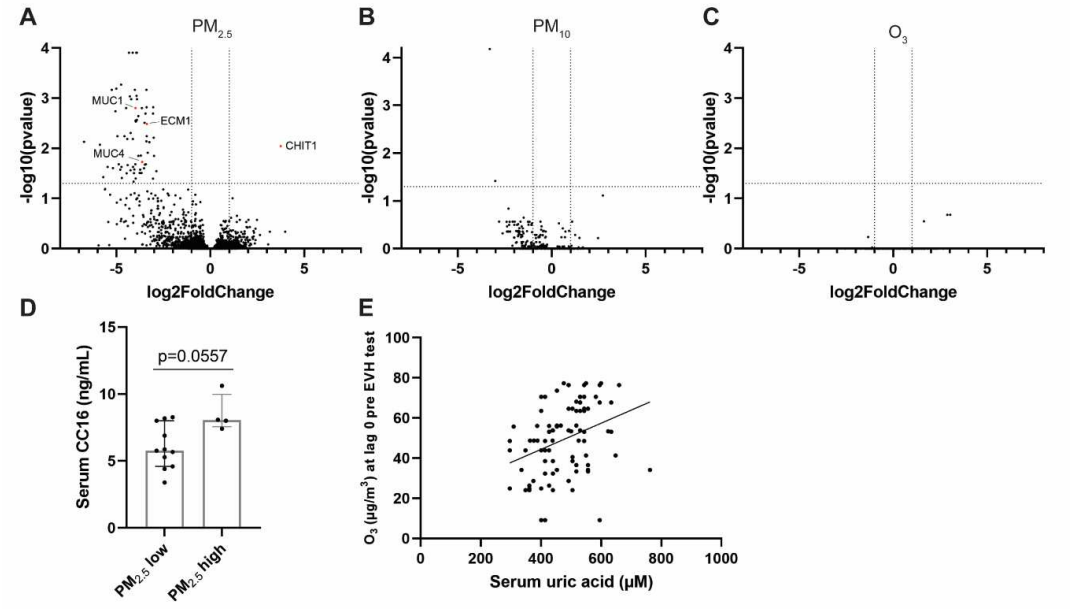

Figure E7: Transcriptomic differences in athletes based on air pollution exposure.

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 for $PM_{2.5}$ low (<10 µg/m^3) vs high (>25 µg/m^3) (A), PM_{10} low (<20 µg/m^3) vs immediate (>20 µg/m^3) (B) (no values above threshold of WHO (50 µg/m^3)) and O_3 low (<25 µg/m^3) vs immediate (O_3>50 µg/m^3) (C) (no values above threshold of 100 µg/m^3), respectively. (D) Serum CC16 levels in athletes exposed to low $PM_{2.5}$ (<10 µg/m^3) compared to high $PM_{2.5}$ (>25 µg/m^3) levels. (Mann-Whitney test). (E) Correlation of serum uric acid levels with O_3 levels at the day of study visit. (spearman correlation).

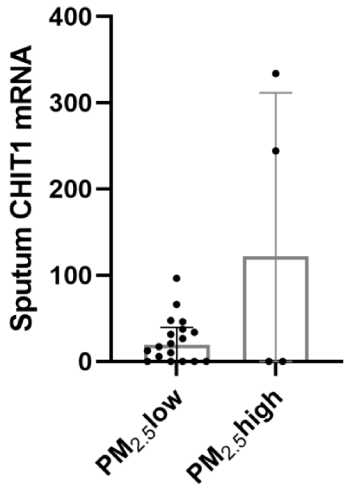

Figure E8: Validation qPCR CHIT1

Remaining RNA for controls athletes exposed to low PM2.5 levels (<10 µg/m^3) (n=18) and PM2.5 high levels (>25 µg/m^3) (n=4) was used to validate obtained results via qPCR. (Mann-whitney test).

Supplementary tables

Table E1: Primer and probe sequences for qPCR

Gene		Sequence
CCL3	FW	5' cct ccc ggc aga ttc cac 3'
	RV	5' gtt agg aag atg aca ccg ggc 3'
	TP	5' ctg act act ttg aga cga gca gcc agt gc 3'
CHIT1	FW	5' fw cct acg act tcc atg gct ctt g 3'
	RV	5' cac agc agc atc cac gtt g 3'
	TP	5' cct cta caa gag gca aga aga gag tgg tgc a 3'
CLDN15	FW	5' att ctg gcc ggt atc tgc g 3'
	RV	5' gcc cag ctc gta ctt ggt tc 3'
	TP	5' atg gtg gcc atc tcc tgg tac gcc t 3'
RPL13a	FW	5' gac cgt gcg agg tat gct g 3'
	RV	5' gca cga cct tga ggg cag 3'
	TP	5' acc gtc tca agg tgt ttg acg gca tc 3'

Table E2: Top 20 genes differentially expressed controls versus athletes

Up	Log2fold change	Down	Log2fold change
HLA-DRB5	3.26	**ZNF385D**	-5.39
MLLT11	2.81	**EML1**	-5.30
PRUNE2	2.53	**RP11-524N5.1**	-5.28
RSAD2	2.24	**FLJ40288**	-5.18
FOLR3	2.12	**RP1-78B3.1**	-5.09
UBBP4	2.07	**RNA5SP378**	-4.98
CCL3L3	2.01	**SLC28A2**	-4.69
RNVU1-19	1.94	**PISRT1**	-4.55
HES6	1.78	**LINC01122**	-4.48
RNU2-46P	1.77	**STATH**	-4.45
BATF2	1.74	**RP11-655M14.13**	-4.44
PLEKHG2	1.65	**HTN3**	-4.38
SASS6	1.57	**AC079779.4**	-4.35
BZRAP1	1.45	**RP11-68D16.1**	-4.21
TNFAIP3	1.29	**SEZ6**	-4.19
CCL3	1.28	**RP11-556H2.1**	-4.17
IER3	1.26	**PAQR9-AS1**	-4.08
GPATCH3	1.26	**FDCSP**	-4.05
ZNF768	1.20	**FERMT1**	-4.00
TNF	1.18	**RP11-151H2.1**	-3.98

Table E3: Characteristics athletes used for comparison EIB- versus EIB+ athletes

EIB	Sport discipline	Gender	Age	Weight (kg)	Length (cm)	Atopic state	FeNO
+	Basketball player	M	15.7	86	192	1	11
-	Basketball player	M	15.3	77	182	1	13
-	Football player	M	14.7	48	165	1	11
+	Volleyball player	F	18.1	71	182	1	166
-	Volleyball player	F	18.0	73	175	1	17
-	Volleyball player	F	17.8	72	186	1	14

Goossens J, *et al. Thorax* 2023;0:1–9. doi: 10.1136/thorax-2022-219651

Table E4: Top 20 genes differentially expressed EIB- versus EIB+

Up	Log2fold change	Down	Log2fold change
RNF212B	8.12	**PRPF39**	-5.49
RIPPLY3	8.01	**NAPSB**	-4.51
PHOX2B	7.95	**KIF20B**	-4.13
NPM1P30	7.68	**IGF1**	-3.69
ONECUT2	7.55	**FTSJ3**	-3.03
KIAA2012	7.50	**C8B**	-2.74
GRIN2B	7.49	**HLA-DQB1**	-2.73
NR2E3	7.46	**PPTC7**	-2.64
RP11-93G5.1	7.44	**PDCD1LG2**	-2.64
CDH4	7.39	**SCFD1**	-2.62
RP4-671O14.7	7.33	**EIF2A**	-2.38
IYD	7.29	**SBDS**	-2.36
RP11-168O16.1	7.27	**FILIP1L**	-2.31
RP11-303E16.6	7.20	**GINM1**	-2.29
RP11-638L3.4	7.20	**CXCL5**	-2.25
RP11-5A11.1	7.14	**GALNT6**	-2.24
RP11-46D6.1	7.09	**ZNF331**	-2.20
CTB-12A17.2	7.08	**KIAA2026**	-2.16
MTCO1P2	6.99	**MAML2**	-2.13
HOTTIP	6.98	**ERC1**	-2.06

Table E5: Correlation table

	Max fall in FEV_1	O_3	Humidity at lag 0	Temp at lag 0	$PM_{2.5}$ at lag 3	PM_{10} at lag 3	Humidity at lag 0	Temp at lag 3	FEV_1%
Max fall in FEV_1	1.000	-.258*	.026	-.072	-.288**	-.291**	.050	-.208	-.079
O_3	-.258*	1.000	-.230*	.480**	.111	.206	-.109	.683**	-.163
Humidity at lag 0	.026	-.230*	1.000	-.401**	.117	-.009	.106	-.302**	-.003
Temp at lag 0	-.072	.480**	-.401**	1.000	-.169	-.026	-.133	.705**	-.181
$PM_{2.5}$ at lag 3	-.288**	.111	.117	-.169	1.000	.955**	.087	.027	.004
PM_{10} at lag 3	-.291**	.206	-.009	-.026	.955**	1.000	-.026	.223*	-.035
Humidity at lag 3	.050	-.109	.106	-.133	.087	-.026	1.000	-.342**	.167
Temp at lag 3	-.208	.683**	-.302**	.705**	.027	.223*	-.342**	1.000	-.198
FEV_1%	-.079	-.163	-.003	-.181	.004	-.035	.167	-.198	1.000

Pearson correlation * Correlation is significant at the 0.05 level (2-tailed), ** Correlation is significant at the 0.01 level (2-tailed).

Table E6: Overview medication use

	Basketball players (n=24)	**Football players (n=38)**	**Volleyball players (n=14)**	**Swimmers (n=14)**	**Controls (n=25)**
Allergy	Cetirizine (n=1)	Levocetrizine (n=1)		Bilastine (n=1)	Cetirizine (n=1)
Upper airways	Mometasone (n=1), nasal spray* (n=4)	Nasal spray* (n=3)		Nasal spray* (n=3)	Mometasone (n=1)
Lower airways	SABA (n=1) ICS (n=1**)	SABA (n=1**, 2), LABA + ICS (n=1**), Montelukast (n=1**)		SABA (n=2), LABA + ICS (n=1**,2), Montelukast (n=2), LABA + ICS + Montelukast + Mucolyticum (n=1**),	
Others	Ibuprofen (n=1), Paracetamol (n=1)	Paracetamol (n=2), Ibuprofen (n=4)	Ivabradine (n=1)		Paracetamol (n=2)

*Subject noted 'nasal spray' but did not know the product's name.

** Subject mentioned in questionnaire to have prior diagnosis of asthma.

Table E7: EIB+ subjects

Nr	Sport discipline	Gender	Atopic state	FEV_1 %	FVC %	TI	Fall 1'	Fall 5'	Fall 10'	Fall 15'
1	Basketball player**	M	1	90	109	72	**-13.35**	**-11.01**	-9.84	**-10.30**
2	Basketball player*	M	1	108	108	86	-6.36	**-13.68**	-7.51	-5.97
3	Football player	F	0	104	114	81	-0.65	**-12.94**	**-15.53**	-1.29
4	Football player	M	0	108	110	85	-2.54	**-27.23**	**-26.49**	-1.49
5	Volleyball player*	F	1	118	117	88	**-10.47**	**-14.96**	**-15.38**	**-11.11**
6	Swimmer	M	0	104	133	68	-9.77	**-11.23**	**-10.60**	**-10.19**
7	Swimmer	M	0	120	133	78	-6.36	**-12.71**	**-12.92**	-8.47
8	Swimmer**	F	0	119	134	79	**-16.58**	**-12.03**	-7.75	-8.02
9	Control	F	1	111	118	78	**-15.74**	**-23.15**	**-19.14**	**-13.58**
10	Control	M	1	105	96	90	-3.85	-9.19	-9.19	**-12.61**
11	Control	M	0	115	108	90	-4.00	-4.75	-7.00	**-10.25**

Maximal fall in FEV1 after the EVH test at different time points in EIB+ young elite athletes.
*Sputum samples of indicated athletes were used for RNA-Seq analysis.
** Subject mentioned in questionnaire to have prior diagnosis of asthma

Table E8: Differential expression analysis between sport disciplines

Gene name	Log2Foldchange	P adjusted
Basketball versus football players		
HELLPAR	-2.6	0.003663
RP11-356K23.1	1.9	0.146214
CFAP45	-2.7	0.197004
TTC25	-1.9	0.197004
NR2F2	0.6	0.267105
HYDIN2	-1.2	0.2999
MIF	-2.9	0.2999
C9orf24	0.7	0.314153
Basketball versus volleyball players		
RPS14	-0.6	0.074010726
CIRBP	-0.4	0.07984527
LAMTOR4	-0.6	0.07984527
Metazoa_SRP	-2.5	0.07984527
SLC37A1	0.9	0.07984527
MT-TT	-1.5	0.090845219
RSAD2	2.1	0.090845219
SMIM3	-1.4	0.090845219
TGM3	-2.4	0.090845219
APOE	-1.2	0.095427481
Football versus volleyball players		
KIAA1551	0.9	0.180642019
RP11-1143G9.4	-5.8	0.196325744
FTH1P3	-2.1	0.258028139
AHNAK	-0.8	0.272184731
SAA1	3.2	0.293974195
MTCYBP18	-3.6	0.606428123
FTH1P1	-3.1	0.677932845
PKN2	0.7	0.677932845
RPS18	-0.6	0.677932845

Top 10 significantly_differentially_expressed genes ordered by adjusted p-value.

Table E9: Top 20 genes differentially expressed $PM_{2.5}$ low exposure (<10 µg/m^3) versus $PM_{2.5}$ high exposure (>25 µg/m^3)

Up	Log2fold change	Down	Log2fold change
CHIT1	3.74	**VSIG2**	-6,73
		ATP12A	-5,88
		RPTN	-5.70
		KRT15	-5.47
		PSCA	-5.25
		TRNP1	-5.19
		CEACAM5	-5.06
		S100A16	-5.01
		FUT3	-4.94
		ALDH1A3	-4.87
		CTGF	-4.80
		TMPRSS2	-4.75
		AIF1L	-4.73
		FCGBP	-4.72
		AQP5	-4.58
		S100A14	-4.48
		PDZK1IP1	-4.46
		S100A7	-4.42
		PRSS8	-4.40
		NDRG2	-4.34

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