Cover by  (my creative and lovely little sister)

KU Leuven
Biomedical Sciences Group
Faculty of Medicine
Department of Microbiology, Immunology
and Transplantation

KU LEUVEN

DOCTORAL SCHOOL
BIOMEDICAL SCIENCES

STUDY OF AIRWAY INFLAMMATION AS A RESULT OF EXTERNAL TRIGGERS INDUCING EPITHELIAL CELL DAMAGE IN NON-ALLERGIC ASTHMA AND EXERCISE-INDUCED BRONCHOCONSTRICTION

Janne GOOSSENS

Jury:

Supervisor: Prof. Dr. Dominique Bullens
Co-supervisor: Prof. Dr. Lieven Dupont
Chair examining committee: Prof. John Creemers
Chair public defense: Prof. Dominique Schols
Jury members: Prof. Dr. Mieke Boon
Prof. Thierry Troosters
Prof. Valérie Bougault
Prof. Dr. Renaud Louis

Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in Biomedical Sciences

June 2023

Dankwoord

De afgelopen 5 jaar heb ik aan deze thesis gewerkt, maar dit deed ik niet alleen. Eerst en vooral wil ik mijn promotor professor Dominique Bullens en copromotor professor Lieven Dupont bedanken.

Dominique, bedankt om me de kans en het vertrouwen te geven om dit doctoraatsproject te starten. Ik kan wel besluiten dat het een zeer gevarieerd onderzoeksproject was: van het leren aansluiten en verplaatsen van gasflessen, opstarten en coördineren van multicentrische klinische studies tot het ontwerpen van T-shirts voor onze meter en peter van sportersstudie. Bedankt voor al je goede raad, vertrouwen, input, fijne babbels en dat ik altijd bij je terecht kon voor vragen, twijfels en steuntjes in de rug. Je jarenlange ervaring binnen immunologie, je enthousiasme en positivisme hebben mijn intereresse voor onderzoek verder gestimuleerd en dit werkte heel motiverend. Professor Dupont, bedankt om me te introduceren op de dienst pneumologie. Ondanks je drukke schema je toch nog tijd kon vrijmaken voor onze maandelijkse meetings, waarbij ik je input enorm apprecieerde. Met jouw ervaring en hulp binnen de pneumologie en humane studies, vormden jij en Dominique een goed begeleidend team tijdens mijn doctoraat.

Graag wil ik mijn interne jury leden bedanken, professor Mieke Boon en professor Thierry Troosters. Dankjewel voor jullie opvolging, opbouwende kritiek, tijd en moeite de afgelopen 5 jaar. I would like to thank my external jury members, professor Renaud Louis and professor Valérie Bougault, for their time to read my manuscript, for the interesting suggestions and their presence during the defense. I would like to thank the chair of my reading committee, professor John Creemers, and the chair of my public defense, professor Dominique Schols for ensuring the evaluation process of this thesis would go smoothly.

Daarnaast wil ik ook professor Ceuppens, professor Schrijvers, professor Hellings, professor Van Gerven en professor Breynaert bedanken voor hun kritische blik en input op de wekelijkse labmeetings.

Graag wil ik alle collega's van de onderzoeksgroep Allergie en Klinische Immunologie bedanken. De afterworks, teambuildings, kerstfeestjes, etentjes, congressen, '#vrijmibo's' en nog veel meer hebben de afgelopen 5 jaar onvergetelijk gemaakt met talloze mooie herinneringen. Anne-Charlotte, ik wil je bedanken dat ik me vanaf het begin van dit avontuur altijd gesteund heb gevoeld door jou. Zelfs na het afronden van je doctoraat kan ik nog steeds bij jou terecht. Dank voor het organiseren van leuke teambuildings als 'moeke' van het labo. Ik waardeer je motiverende en trouwe woorden: 'Komt wel (dik) in orde!'. Liselot, Wout en Eva: we zijn samen aan dit avontuur begonnen in het labo. Ik heb genoten van onze leuke tijd samen op congressen in Lissabon en Praag. Wout en Eva, jullie hebben me het goede voorbeeld gegeven met jullie succesvolle thesisverdedigingen. Liselot, bedankt om me te laten proeven van het West-Vlaams (waarvan ik nog steeds niet veel van begrijp) en voor ambiance te zorgen. Ik weet zeker dat je nog uitstekend zal doen! An-Sofie, dankjewel voor

de ontspannende babbels op onze bureau, je was een fantastische buurvrouw. Inge, An-Sofie en Paulien, jullie wisten onze bureau tot een ware ‘stepstalling’ om te vormen en brachten een geweldige sfeer. Lisa, jij kan natuurlijk niet ontbreken in onze bureau, dankjewel voor je hulp, die je met een lach deed. Inge, dankjewel dat ik bij jou terecht kon voor een luisterend oor, onze treinritjes waren altijd prettig! Birgit, heel erg bedankt voor het introduceren van fijne borrels en Backie in ons labo. Samen met An-Sofie, Tine en Paulien, heb je me overtuigd en zijn we nu trouwe ‘BeReal vriendjes’. Ik kijk al uit naar al jullie volgende dagelijkse avonturen! Tine, dankjewel voor het delen van alle ‘Roosje liefde’ en de quote ‘If roosje can dream it, you can do it’. Ik kijk al uit naar het puppy bezoek van Wilson. Ook erg bedankt aan de nieuwere ‘garde’ van het labo: Emma en Willem. Jammer genoeg pasten we niet allemaal meer samen in de bureau, maar desalniettemin wil jullie graag bedanken voor alle fijne momenten die we samen hebben gehad en ben blij dat de toekomst van het labo in goede handen is met jullie veelbelovende resultaten en projecten! PS Ik ben nog steeds beschikbaar als er een tweede editie komt van de BBQ. Toon, bedankt dat je mijn 'mast-cel vriendje' bent geworden. Het heeft me enorm geholpen om mijn haat-liefde verhouding met deze cellen met je te kunnen delen. Je wist elke middagpauze op te fleuren met interessante weetjes en indrukwekkende imitaties. Ook wil ik je bedanken voor de tijd die je hebt vrijgemaakt om met mij mee te gaan naar sportscholen in Vlaanderen. Hiervoor wil ik ook Kasran, Frederik en Wout hartelijk bedanken. Ook dankjewel aan de (ex-)postdocs van het labo Brecht, Katleen en Lies. Dankjewel om me welkom te heten in het labo en me tijdens dit traject te ondersteunen. Ook wil ik alle andere collega’s: Alba, Cecilia en Dorien bedanken en hun verder veel succes wensen met hun doctoraat! Tenslotte ook een dankjewel aan onze labo visjes Blotje en Elisa, voor het leuke gezelschap vroeg in de ochtend en houd een oogje in het zeil bij deze zotte bende phd’ers blub blub blub.

Bedankt aan de leden van team ‘astma en EIB’ waarmee we maandelijks meetings hebben. Tatjana, dankjewel om me te introduceren in het onderzoek van de sporters en ons uitje naar de gymnasten in Gent. Nora, het was fijn met jou samen te werken. Nog veel succes bij het starten van de humane studies en je opleiding geneeskunde!

Ellen, jou wil ik bedanken voor alle fijne babbels van ’s morgens vroeg onderweg naar de topsportscholen of tijdens de wachtstappen van de immunoassays. Het was een eer om met jou alle topsportscholen in Vlaanderen af te schuimen. Bij vragen kon ik altijd bij jou terecht en ook culturele uitstapjes zoals scala en synchroon zwemmen hoorden erbij. Zo mogen er zeker nog volgen. Jonathan, dankjewel voor alle hulp bij flow cytometry en voor het bijstaan bij allerhande technische vragen. Lieve, dankjewel voor alle praktische zaken in het labo mee op te volgen en onze lunches in de UZ cafetaria pre-Covid. Ook wil ik Sabien, Caroline en Marleen bedanken voor al hun hulp en toegankelijkheid.

Daarnaast kon ik ook rekenen op hulp van fijne en getalenteerde kinderartsen. Sophie, dankjewel voor al je enthousiasme en input in het project. Jammer genoeg viel de start van onze samenwerking juist in corona, maar vanaf toen kreeg ik al motiverende support van jou. Ik wens je veel succes toe met PeAACH in Hasselt. Josefien, jij werd het laatste jaar mijn vast

maatje waarop ik steeds kon rekenen. Samen met jou het 'Childhood Immunology Symposium' organiseren was een groot plezier! Ons tripje samen naar Hamburg is een mooie afsluiter van onze uitstekende samenwerking.

Dankzij het FWO-TBM project van de sporters kon ik samenwerken met een mooi team van artsen over verschillende centra. Marc Raes en Jasmine Leus, jullie zou ik graag willen bedanken als copromotors van dit project in Hasselt en Gent. Dankjewel voor jullie enthousiasme tijdens meetings van het TBM project, de fijne samenwerking en input in manuscripten. In elk centrum kon ik ook rekenen op een team van enthousiaste medewerkers wie ook hun steentje bijdroegen aan het project: dankjewel Marie-Paule, Sanne, Evelien, Linsey, Charlotte, Elien en Delphine. Ook heel erg bedankt aan de leden van de begeleidingscommissie. Jullie zorgden mee voor een vlot verloop van het project, manieren om jonge atleten te rekruteren en oplossingen tijdens de Covid-19 pandemie. Eveneens wil ik Jana Raman en Louis Croenen bedanken voor het meter/peterschap van onze studie en zo te helpen aan de rekrutering van onze grote groep jonge atleten.

Geert Celis, jou wil ik bedanken voor alle hulp, raad en vertrouwen tijdens de longfunctiemetingen en klinische onderzoeken die ik mocht uitvoeren op jullie dienst. Daarnaast wil ik ook alle mensen van de dienst longfunctiemetingen bedanken voor de assistentie bij de onderzoeken.

Bedankt aan alle bachelor/master studenten die me de afgelopen 5 jaar geholpen hebben met verschillende project: Camille, Lisa, Imke en Liesbeth.

'Een band gaat nooit stuk, Al heb je een ongeluk. Je ziet maar, ze hebben elkaar. Ze krijgen het telkens weer voor mekaar'. Graag wil ik al mijn vriendinnen bedanken waarop ik kon rekenen voor alle leuke en ontspannende momenten. Bedankt aan de vriendinnen van Biomedische wetenschappen (ook gekend als de BMW Queens): Eva, Dorien, Michelle, Daphne, Femke en Ann. Voor de nodige ontspanning tijdens het studeren en de onvergeetbare reisjes en tripjes achteraf. Dat er nog veel mogen bijkomen! Ook al wonen we niet bij elkaar in de buurt, onze vriendschap kent geen grenzen. Natuurlijk ook aan alle Chics van Heist, die me nauw hebben gevolgd en vanaf de middelbare school hebben gesteund. Jullie voorspelling dat ik ooit een beroep in witte jas zou uitoefenen is in ieder geval uitgekomen. En natuurlijk ook mijn favoriete Amigo's voor al hun steun! Ik kon rekenen op motiverende en lieve briefjes, leuke feestjes, wandelingen, weekends, en nog veel meer. Dit werd enorm gewaardeerd. Ik ben blij dat ik jullie eindelijk kan verwelkomen op die receptie.

Moeke en vake, jullie wil ik bedanken voor wat jullie als de beste kunnen: in mij geloven en me steunen. Zonder het vertrouwen en de warmte die jullie me hebben meegegeven zou ik hier niet staan. Vake, jij hebt zelfs zelf bijgedragen aan dit mooie resultaat. Dankjewel dat je samen met mij oplossingen hebt gezocht voor al mijn computer gerelateerde vragen en voor de gemeentes die we samen een gridnummer hebben gegeven. Moeke, je bleef altijd heel geïnteresseerd in wat ik deed en zorgde voor liefdevolle steun: bij momenten om te vieren,

zoals de heerlijke taart bij mijn final doctoral plan of een knuffel bij momenten waarop alles even tegen zat.

Mirthe, dankzij jou kon ik rekenen op een mooi voorblad op deze thesis. Je bent mijn kleine zus waarvan ik veel geleerd heb en waarop ik apetrots kan zijn. Ik wens je alle liefde toe samen met Pepijn, welke we volgend jaar gaan vieren op jullie huwelijk.

Ook kan ik rekenen op een prachtige schoonfamilie waar ik me altijd welkom voel en er op donderdag een heerlijke maaltijd klaar staat die ons allemaal bij elkaar brengt. Het afgelopen jaar hebben we geleerd wat echt belangrijk is in het leven. Bovendien heeft de komst van mijn eerste neefje, Mon, me al veel vreugde en liefde gebracht.

Ook aan alle andere familie dankjewel voor alle warme en ontspannende momenten.

En niet te vergeten ook aan jou Vinny, mijn partner, lief en beste vriend, een hele dikke merci! Jij had de eer had om alles vanop de eerste rij mee te volgen en kan hierbij bevestigen dat er bloed, zweet en tranen in dit PhD verhaal zitten. Ik heb je zelfs kunnen overtuigen om zelf mee proefkonijn te zijn en in levende lijve te ondervinden waarmee ik allemaal bezig ben. Dankjewel om dit allemaal met mij te willen delen van begin tot eind. En dit in combinatie met de bouw van eigen project in de Kasteelstraat, waar we nu samen kunnen thuis komen. Ik ben benieuwd wat nog komen zal, maar met jou erbij zit dat zeker goed!

Op naar het volgende avontuur!

Janne

Table of contents

List of abbreviations

ACOS	Asthma COPD overlap syndrome
ACQ	Asthma Control Questionnaire
ACT	Asthma Control Test
AERD	Aspirin-exacerbated respiratory disease
AhR	Aryl hydrocarbon Receptor
AHRR	Aryl hydrocarbon receptor repressor
AQUA	Allergy Questionnaire for Athletes
ATS	American Thoracic Society
BAL	Bronchioalveolar lavage fluid
BC	Black carbon
BMI	Body mass index
CaCC1	Ca^{2+}activated Cl- channel 1
CC16	Club cell protein 16
CCL	C-C motif ligand
CD	Cluster of differentiation
CLDN	Claudins
CMA1	Chymase
COPD	Chronic obstructive pulmonary disease
CPA3	Carboxypeptidase A3
CRS	Chronic rhinosinusitis
CRTH2	Prostaglandin D2 receptor 2
CXCL	C-X-C motif ligand
CYP1B1	Cytochrome P450 Family 1 Subfamily B Member 1
CysLT	Cysteinyl leukotrienes
DAG	Diacylglycerol
DAMP	Damage-associated molecular pattern
DEG	Differentially expressed gene
DGC	Density gradient centrifugation
DTT	Dithiothreitol
EIB	Exercise-induced bronchoconstriction
ERS	European Respiratory Society
EVH	Eucapnic voluntary hyperventilation
FBS	Fetal bovine serum
FcγR	Fc receptor for IgG
FcεRI	High-affinity Fc receptor for IgE
FDA	U.S. Food and Drug Administration
FDR	False discovery rate
FeNO	Fractional exhaled nitric oxide
FESS	Functional endoscopic sinus surgery
FEV_1	Forced expiratory volume in 1 second
FGF	Fibroblast growth factor
FVC	Forced vital capacity
G-CSF	Granulocyte colony-stimulating factor
GERD	Gastroesophageal reflux disease
GINA	Global Initiative of Asthma

GM-CSF	Granulocyte-macrophage colony stimulating factor
GO	Gene Ontology
GSEA	Gene set enrichment analysis
HMGB1	High-mobility group box 1
HDM	House dust mite
ICS	Inhaled corticosteroids
IFN	Interferon
IgE	Immunoglobulin E
IL	Interleukin
IL1RL1	Interleukin 1 receptor-like 1
ILC	Innate lymphoid cell
IMS	Immunomagnetic selection
IOC	International Olympic Committee
IP_3	Inositol triphosphate
IPA	Ingenuity Pathway Analysis
IQR	Interquartile range
IRCEL	Belgian Interregional Environment Agency
LABA	Long acting beta-receptor agonists
LACI	Allergy and Clinical immunology Research Group
LAG	Clinical Laboratory (Laboratoriumgeneeskunde)
LDL	Low density lipoprotein
LT	Leukotriene
LTB4	Leukotriene B4
LTC4	Leukotriene C4
MAPK	Mitogen-activated protein kinase
MC	Mast cell
MCH	Major histocompability complex
MC_T	Tryptase positive mast cells
MC_{TC}	Tryptase and chymase positive mast cells
MRGPRX2	MAS related GPR family member X2
MVV	Maximal voluntary ventilation
NF-κB	Nuclear factor κB
NGF	Nerve growth factor
NK1/2	Neurokinin 1 or 2 receptor
NKA	Neurokinin A
NLR	Nod-like receptors
O_3	Ozone
OCLN	Occludin
OSAS	Obstructive sleep apnoea syndrome
PAF	Platelet-activating factor
PAMP	Pathogen-associated molecular pattern
PAR2	Protease-activated receptor 2
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PC_{20}	Provocative concentration inducing a 20% decrease in FEV_1
PG	Prostaglandin
PGD2	Prostaglandin D2
PGE2	Prostaglandin E2

PIP2	Phosphatidylinositol 4,5-bisphosphate
PM	Particulate matter
PM_{10}	Particulate matter ≤ 10 µm
$PM_{2.5}$	Particulate matter ≤ 2.5 µm
PNEC	Pulmonary neuroendocrine cell
ppb	Parts per billion
PRR	Pattern-recognition receptor
RMI	Royal Meteorological Institute of Belgium
RNA-Seq	RNA sequencing
ROS	Reactive oxygen species
SABA	Short acting beta-receptor agonists
SCF	Stem cell factor
SN	Supernatant
SP-D	Surfactant protein D
SPT	Skin prick test
ST2	Interleukin 1 receptor like 1 (IL-33 receptor)
TGF-β	Transforming growth factor-beta
Th1	Type 1 T helper cell
Th17	Type 17 T helper cell
Th2	Type 2 T helper cell
TI	Tiffeneau index
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TPSAB1	Tryptase Alpha/Beta 1
Treg	Regulatory T cell
TRPA1	Transient receptor potential ankyrin 1
TRPV1	Transient receptor potential vanilloid 1
TSLP	Thymic stromal lymphopoietin
TXB2	Thromboxane B2
VCD	Vocal cord dysfunction
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
ZO	Zonula occludens

Chapter 1

General introduction

1. The airways

Human airways are ventilated by thousand liters of air per day over roughly 100 m^2 surface of the lung, accordingly being the largest organ with direct contact to the atmospheric environment (1). The human respiratory system is composed of two main parts: the upper respiratory tract, which includes the nasal cavity, pharynx and larynx, and lower respiratory tract (figure 1). The latter can be divided into two distinct zones: the conducting zone and the respiratory zone each with their distinct purpose, namely the transmission of gases and gas exchange, respectively (2). The respiratory zone comprises the respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli (2). On the other hand, the conducting zone includes the trachea, bronchi and extends up to the terminal bronchioles (figure 1). Chronic lung diseases, such as asthma and COPD, are a result of inflammation or obstruction within the lungs (3) and pose significant challenges to the global health system (4).

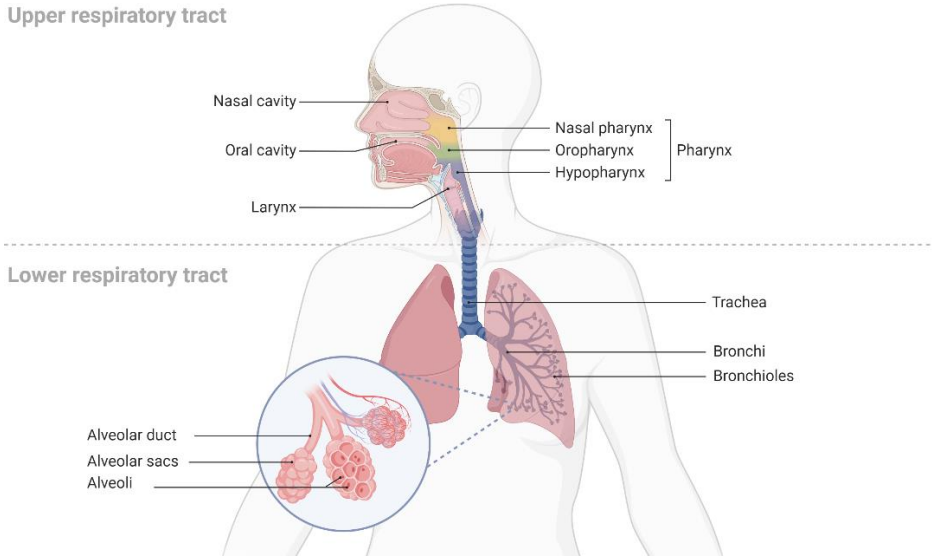

Figure 1: Human respiratory system. (Created with Biorender.com)

1.1. Asthma

Asthma is a chronic inflammatory disease, which is characterized by reversible airway obstruction (5). Up to 262 million people worldwide are estimated to suffer from asthma in 2019, according to WHO (6). Recurrent wheezing, chest tightness, cough and shortness of breath are all common symptoms of asthma (5). Patients with asthma experience these symptoms following exposure to (non-)specific stimuli such as pollutants, cigarette smoke, allergens, viruses, pathogens, cold air, etc (7). An asthma diagnosis is based on identifying both a characteristic pattern of respiratory symptoms and variable expiratory airflow limitation (5).

The strongest risk factors for developing asthma include a genetic predisposition and environmental exposure to external triggers (8). Atopy, the genetic predisposition to develop antibodies against allergens, is a well known risk factor to develop allergic asthma. As mucus overpoduction is a clinical feature of asthma, Ca^{2+} activated Cl^- channel 1 (CaCC1) gene has been described to play an important role (9). Moreover, genome-wide association studies revealed asthma related genes including disintegrin and metalloproteinase 33 (ADAM33) and immune cell marks such IL-33, IL1RL1, thymic stromal lymphopoietin (TSLP), but explain only a part of the hereditary component of asthma (10). Besides, asthma development and exacerbations are also significantly influenced by environmental risk factors like viral infections, environmental allergens, air pollution, tobacco smoke, and lifestyle factors like diet, and exercise (11).

Heterogeneity of asthma

For decades asthma has been considered to be a single disease, but evolved into a syndrome with enormous heterogeneity. Asthma can vary in underlying mechanisms, age of onset, severity and treatment response (8). In literature, different asthma clusters are described based on mechanistic differences (endotypes) and clinical characteristics (phenotypes) to in the end obtain more insights in their differences and lead to tailored treatment options. Based on age, asthma can be subdivided into childhood-onset asthma, which has generally an allergic orgin, and adult-onset asthma. Subsequently, the distinction between allergic (also called extrinsic) and non-allergic asthma (also called intrinsic asthma) is classically made in clinics (figure 2).

Allergic asthma is typically characterized with an antigen driven immune response and eosinophilic inflammation driven by T helper (Th2) lymphocytes and type 2 cytokines (Interleukin (IL)-4, IL-5, IL-13). It is induced by sensitisation to environmental allergens, leading to activation and differentiation of allergen-specific Th2 cells, inducing proliferation of immunoglubin E (IgE) producing B cells (figure 2). The dendritic cells are antigen presenting cells, capturing foreign particles and present them to naïve T cells in the lymph nodes inducing Th2 maturation. Matured T cells will migrate towards the aiways where they are activated by epithelial derived cytokines, such as TSLP, IL-33 and IL-25. These activated Th2 cells will produce typical type 2 cytokines: IL-4, regulating IgE synthesis by B-cells; IL-5, inducing eosinophilic influx; and IL-13, inducing globlet metaplasia and bronchial hyperreactivity (12).

Mast cells will be activated by an IgE mediated pathway. In addition, the recently described innate lymphoid cells (ILCs) can be of particular importance in airway inflammation in asthma (13). Three different subsets are described: ILC1s, ILC2s and ILC3s, lacking the adaptive antigen receptors, are the innate counterparts of T lymphocytes (14). ILC2s are important players in Th2-high inflammation. In response to epithelial damage by external factors and subsequent release of alarmins (IL-25, IL-33 and TSLP), they are also able to induce cytokine-dependent inflammation and airway hyperresponsiveness (15). This might explain a severe eosinophilic inflammation in the absence of classical Th2-mediated allergic response. High number of ILC2s are identified in both allergic eosinophilic inflammation and non-allergic eosinophilic airway inflammation in asthma caused by pollutants and microbes (12, 13, 16).

Non-allergic asthma includes subsets of subjects with asthma in whom allergic sensitisation cannot be demonstrated. The inflammatory reaction in non-allergic asthma phenotype is, on the other hand, mediated by Th17-type and/or Th1-type cytokines (17), or can also be mediated by Th2-type cells with eosinophilic inflammation as a result, like mentioned above. The Th1 response includes the cytokines IFN-γ and TNF-α, which are associated with corticosteroid resistance (18). TNF-α is mainly produced by macrophages and mast cells and it promotes neutrophil chemotaxis. Th17 immune cells produce IL-17, IL-21 and IL-22. IL-17 will recruit neutrophils and activation of them will occur via production of IL-6, G-CSF, GM-CSF, IL-8, CXCL1 and CXCL5 from airway epithelial cells (19). Furthermore, activated ILC3s by IL-1β or IL-23, are also a source of IL-17 (13).

Non-Th2 asthma includes relatively late–onset, obesity-associated asthma as well as smoking-related and neutrophilic asthma, and asthma with minimal inflammation in the affected patients. Later-onset eosinophilic asthma without traditional allergic elements is considered more likely to be severe. Aspirin-exacerbated respiratory disease (AERD) is a recognized subphenotype of this late-onset phenotype. Regarding the type of airway inflammation following distribution is described in literature: about 41% of adult asthmatics have eosinophilic inflammation, 40% have pauci-granulocytic inflammation, 16% have neutrophilic inflammation, and 3% have mixed granulocytic inflammation (20, 21).

To resume, there is a complex interaction between airway epithelium and immune cells with various aspects of innate and adaptive immunity, in the initiation and continuation of asthma (figure 2).

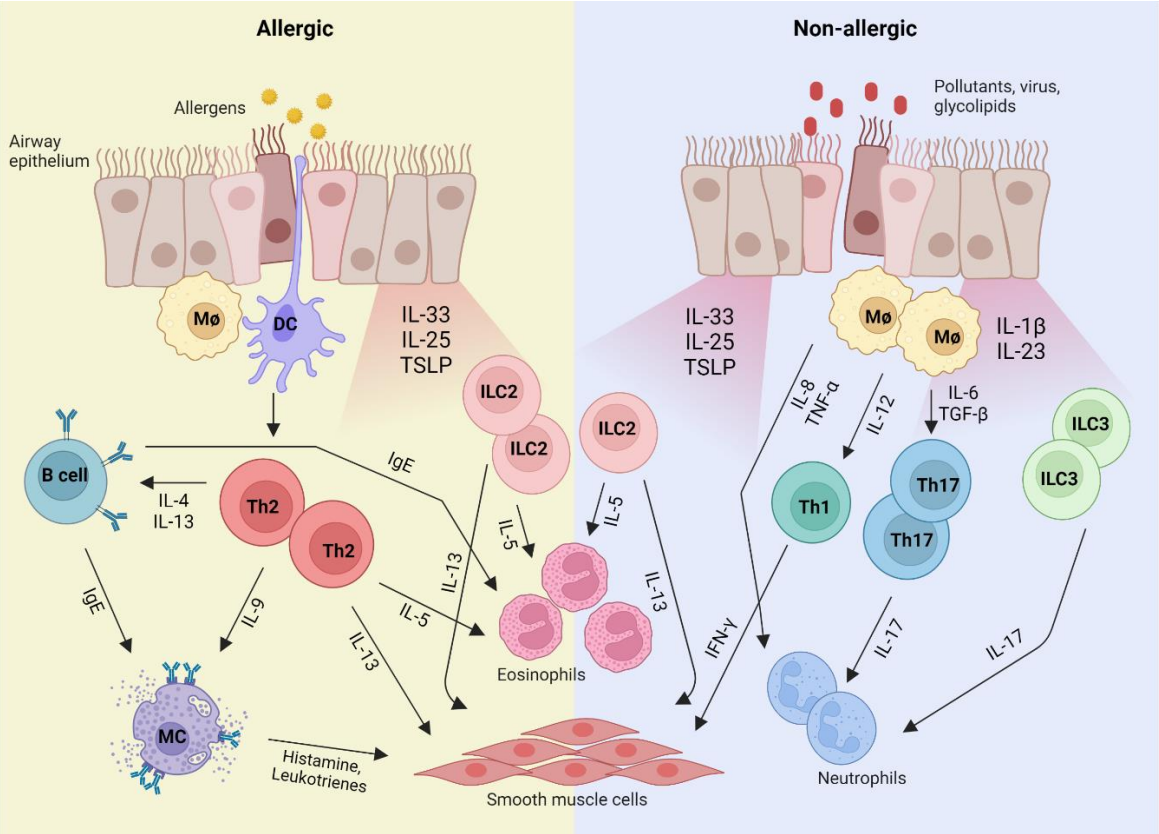

Figure 2: Immunologic pathways involved in allergic and non-allergic asthma. The airway epithelium is important in both type 2 and non-type 2 inflammation, due to allergic and non-allergic triggers (e.g. pollution, virus and glycolipids). Adapted from Brusselle *et al.* (2013) (22) and Boonpiyathad *et al*. (2019) (23). (Created with Biorender.com)

Asthma treatment

A stepwise guideline has been developed by GINA (24). The cornerstone of asthma treatment *anno* 2022 remains the use of inhaled corticosteroids (ICS) with or without short/long acting beta-receptor agonist (SABA/LABA). ICS have an anti-inflammatory effect, through inhibition of induction of Th2 cells and of Th2-cytokine production (11). β2-adrenergic agonists have a bronchodilatory effect through the β2-adrenergic receptor in bronchial smooth muscle cells, which results in smooth muscle relaxation and bronchodilation (12). If asthma control is not reached with above guidelines, physicians consider treatment with a so called 'biological' to improve asthma symptoms. Several biologicals are already available on market to treat asthma: omalizumab (anti-IgE), mepolizumab (anti-IL-5), reslizumab (anti-IL-5), benralizumab (anti-IL5-receptor), dupilumab (anti-IL-4α-receptor) and tezepelumab (anti-TSLP) (25). Based on the heterogenity of asthma and current availability of biotherapeutic agents, it is important to introduce personalized medicine for patients who do not respond to conventional asthma therapy. Potential responders can be identified by looking at specific biomarkers.

1.2. The epithelial barrier

As mentioned above, epithelial cells are a major culprit in asthma. Nowadays, the respiratory epithelial barrier is well accepted to be more than just a physical barrier with the external environment. The epithelial barrier is built up by different cell types, varying in composition throughout the human respiratory tract: ciliated epithelial cells, non-ciliated mucous goblet cells, club cells (initially known as clara cells), basal cells and the novel neuroendocrine and chemosensory cells (tuft cells) and ionocytes (26, 27). Epithelial cells play an active role to maintain homeostasis and initiate immune reactions when necessary (28). Subsequently, epithelial cells contribute to both allergic and non-allergic airway inflammation.

Most of environmental molecules will be trapped in the airway surface liquid (ASL) consisting of mucus layer and cleared via ciliary movements. Beneath this, the ASL includes the periciliary layer, which surrounds the ciliated epithelial cells and contains proteins, glycoproteins, lipids, peptides, ions and water. The production and balance between mucin proteins, like Muc5AC and Muc5B, are key elements to maintain healthy mucus levels (29). Dysregulation of mucin production has been associated with asthma and chronic rhinosinusitis (26). Active ion transport across epithelial cells regulates the ASL thickness (30). Changes in ion transport have been proposed in asthma pathogenesis (31). Surfactant proteins, produced by club cells, cover the epithelial layer, reducing surface tension. Surfactant dysfunction has also been described in asthma (32). The physical barrier arises from tight cell-cell adhesion complexes of neighbouring epithelial cells (figure 3). Tight junctions are the most apically located intercellular junctions and are important for controlling paracellular permeability, including ion transport and limiting the transport of macromolecules (33). Tight junctions are composed of several transmembrane proteins, such as claudins, occludin, tricellulin, junctional adhesion molecules and cytoplasmic proteins such as zonula occludens (ZO)-1, 2 and 3. Adherens junctions are located directly below tight junctions and initiate and mediate cell-cell adhesion. The principal proteins of adherence junctions are α-catenin, β-catenin and E-cadherin. They furthermore regulate the actin cytoskeleton, intracellular signalling and transcriptional regulation and initiate and stabilize tight junctions. Desmosomes attach to adjacent cells as spot-like adhesions to help maintain cell-to-cell adhesion. All these junctional molecules together form a tight epithelial barrier restricting the passage of external particles such as air pollutants, allergens, pathogens, etc. Disturbance of this structure is considered to be a central element in the pathogenesis of asthma (34).

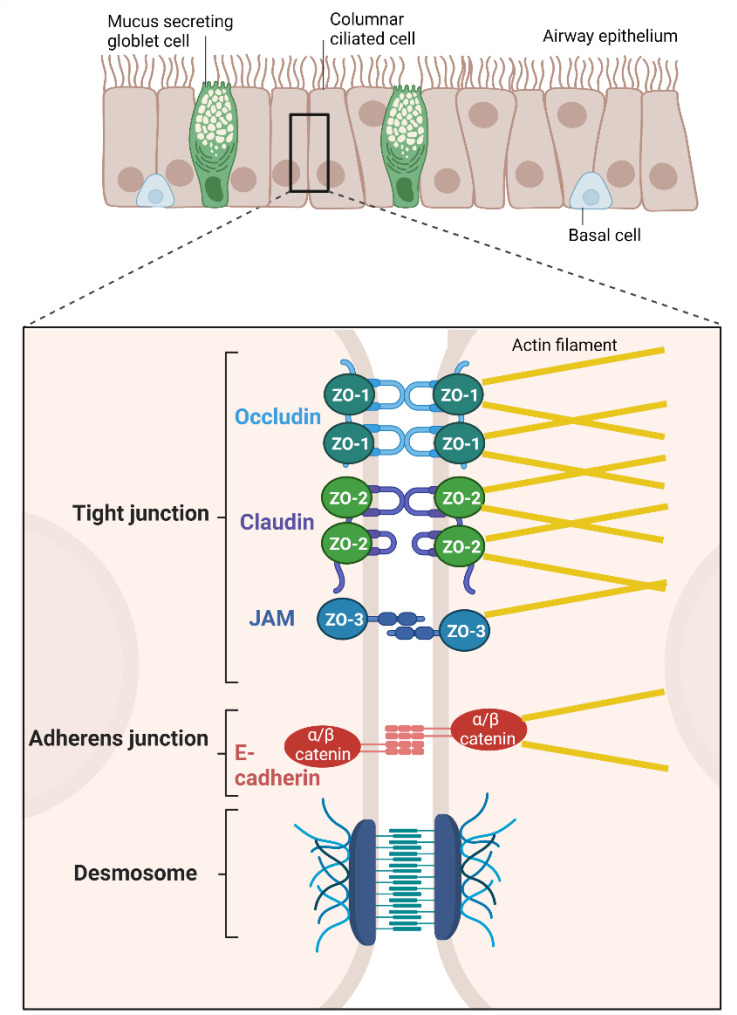

Figure 3: Organisation of intracellular junctions in the airway epithelium. Adapted from Steelant et al. 2020. (26). (Created with Biorender.com)

Moreover, epithelial cells are also part of the innate immune system, being the first line of immune defence to protect the airways from the environment (35). Rapid recognition of both exogenous and endogenous stressors is essential, therefore epithelial cells express proteinase-activated receptors (PAR) and multiple pattern recognition receptors (PRR) such as toll-like receptors (TLRs) and nod-like receptors (NLRs) to sense pathogen-associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs), releasing upon cellular damage and apoptosis (36). Activation of these receptors lead to the release of cytokines, chemokines and antimicrobial peptides, attracting and activating innate and adaptive immune response as dendritic cells, macrophages, mast cells, T- and B-cells, neutrophils and eosinophils. Specifically, DAMPs such as uric acid and high-mobility group box 1 (HMGB1) or alarmins such as TSLP, IL-33 and IL-25 can be released (figure 2).

In addition, it is well recognized that the airways are highly innervated by both nociceptor sensory neurons and cholinergic neurons, enabling interactions with epithelial cells. Chemosensing is modulated via a variety receptors and ion channels such as transient receptor potential vanilloid 1 (TRPV1) and ankyrin 1 (TRPA1) (37). Single cell genomics studies even discovered the pulmonary neuroendocrine cells (PNECs) (27). They are the only innervated epithelial cell type, containing neuropeptide and neurotransmitters. This combination of PNECs and direct neuronal innervation may represent a way in which airways respond to external triggers in a neuro-mediated pathway.

1.3. Mast cells

Since mast cells are located close to the epithelial barrier, they are thought to respond to external stimuli, resulting in recruitment and activation of other immune cells. The role of mast cells has been mainly studied in the pathogenesis of airway allergies such as asthma with focus on IgE-mediated reactions. However, recent techniques revealed novel immunological and functional properties of mast cells in and beyond allergic diseases, with also a role for non-IgE mediated activation pathways. Therefore, we elaborate more on the 'old' mast cell in this part of the thesis.

Mast cells are multifunctional and granulated tissue-resident cells located with high numbers close to interfaces interacting with the external environment such as lung, digestive tract and skin. Therefore, they are thought to also respond to external stimuli. Accordingly, mast cells play an important protective role and participate in defence against pathogens, immune tolerance, wound healing and homeostasis (38). Conversely, mast cells can also play an important role in allergic reactions and pathophysiology of different diseases such as mastocytosis, rheumatoid disease and atherosclerosis (38). Mast cells are considered to be part of both the innate and adaptive immune system. They form a heterogenous population with differences described in their development, mediator content and function. Mast cells are derived from committed hematopoietic progenitor cells in the bone marrow. These $CD34^+$ progenitor cells circulate in the blood stream and migrate into peripheral tissues to differentiate in functional mast cells under the influence of various factors such as IL-3, IL-4, IL-9, IL-10, IL-33, CXCL2 and TGF-β (38, 39). Their mediators are classically divided as pre-stored or *de novo* synthesized. The stored mediators are rapidly released and include mediators such as serotonin, histamine and enzymes such as tryptase, carboxypeptidase A

and chymase (38)(figure 4). In contrast, *de novo* synthesized mediators are produced upon mast cell stimulation, like lipid mediators including leukotrienes (LTs), prostaglandins (PGs) and platelet-activating factor (PAF) (38)(figure 4). Another class includes cytokines, chemokines and growth factors such as IL-1β, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, CCL2, CCL5, GM-CSF, TGF-β, and fibroblast growth factor (FGF) (38)(figure 4). In humans, mast cells are typically classified based on the composition of produced serine proteases, into tryptase and chymase positive mast cells (MC_{TC}) and only tryptase positive mast cells (MC_T) (40). Based on immunohistochemistry MC_{TC} have been labelled in connective tissue such as the airway smooth muscles, while MC_T are predominantly found at mucosal surfaces such as the bronchial epithelium. With the development of more sensitive techniques, this traditional classification system with associated tissue shifted towards more heterogenous mast cell subsets in which distribution is closely linked with tissue microenvironments (41).

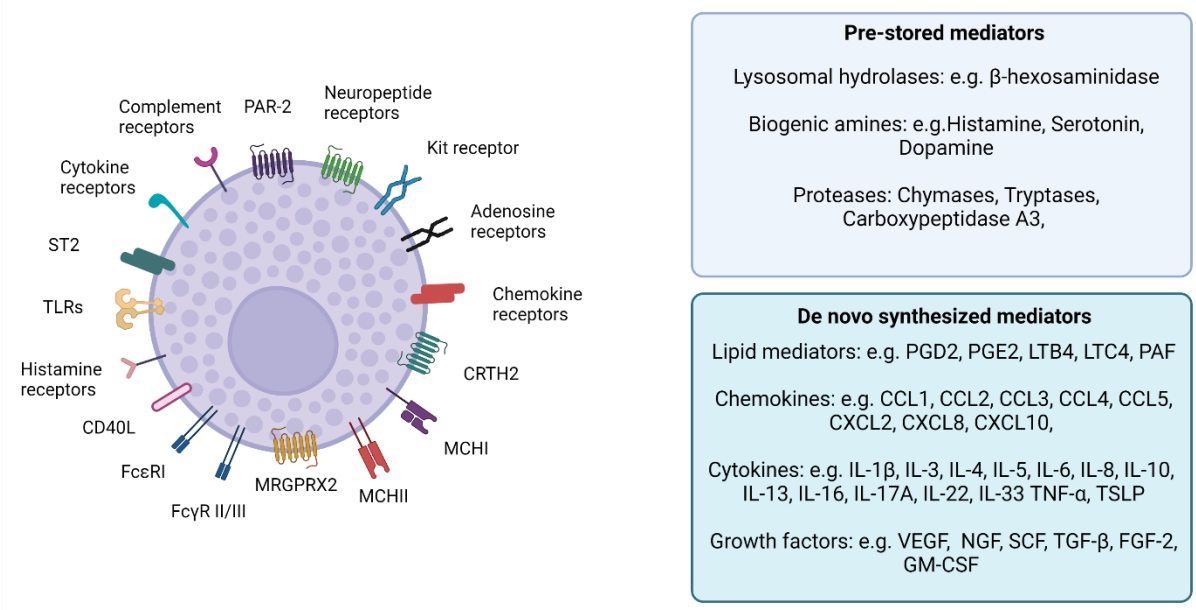

Figure 4: Overview of main mast cell surface receptors and mediators. Adapted from Elieh et al. 2020 (38). CRTH2, Prostaglandin D2 receptor 2. FcεRI, High-affinity Fc receptor for IgE; FcγR, Fc receptor for IgG; MCH, Major histocompatibility complex; MRGPRX2, MAS related GPR family member X2; PAR2, Protease-activated receptor 2, ST2, Interleukin 1 receptor like 1 (IL-33 receptor). (Created with Biorender.com)

Mast cell activation

Mast cell activation and degranulation is best studied in allergic asthma, known as the classical IgE-dependent activation. Cross-linking of IgE with the high affinity receptor for IgE (FcεRI) after inhalation of aeroallergens will trigger mast cell activation. Upon antigen binding, the FcεRI receptor forms dimers, initiating cytoplasmic signalling. In short, activation of tyrosine kinases will lead to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol trisphosphate (IP_3) and initiating of mitogen-activated

protein kinase (MAPK) cascade. The MAPK pathway induces transcription factor activation and subsequent cytokine and eicosanoids (such as PGD2 and LTC4) production. Ca^{2+} mobilization is triggered by the second messenger IP_3, leading to Ca^{2+} influx. Influx of Ca^{2+} is critical for the release of both stored and newly generated mediators.

In addition, mast cells also express a variety of receptors enabling mast cell IgE-**in**dependent activation by diverse molecules: adenosine, complement, cytokines (e.g. TSLP, TNF-α, IL-33) proteases (e.g. tryptase), neuropeptides and TLR ligands (42)(figure 4). Growing evidence suggests critical roles for epithelium-derived cytokines in regulation of inflammatory responses by mast cells in either a direct or indirect way in other subtypes of asthma too (43–45). Furthermore, the newly identified mast cell receptor MAS-related G protein-coupled receptor X2 (MRGPRX2) may play an important role in IgE-independent mast cell activation in asthma. MRGPRX2 is a seven transmembrane, nonselective and low-affinity receptor, and recognizes a wide range of cationic ligands, including FDA approved drugs (e.g. morphine, vancomycin), host defence proteins, venoms (e.g. mast cell degranulating peptide) and neuropeptides such as substance P and hemokinin-1 (46, 47). Downstream signalling from MRGPRX2 involves the activation of Gi protein, inducing activation of MAPK and phospholipase-C pathway, releasing preformed and *de novo* synthesized mediators (48). MRGPRX2 mediated activation seems to be more rapid, but also more transient compared to IgE mediated activation (49). The expression of MRGPRX2 was found to be significantly upregulated in asthma lung mast cells compared to non-asthma lung mast cells (50).

Interaction with immune cells

The activity of many cell types is regulated by activated mast cells, as mast cells express multiplicity of cell surface receptors and can release a whole cascade of different mediators (figure 4)(51). These interactions are important in the defence against pathogenic microorganisms including viruses, parasites and bacteria and tissue repair mechanisms.

Regarding the innate immune system, dendritic cells and mast cells are both located close to environmental interfaces. Direct communication between both cells occurs via formation of synapses, promoting the exchange of mast cell internalized specific antigen to the surrounding dendritic cells for T cell activation (52). Histamine increases IL-10 and decreases IL-12 production by mature dendritic cells, resulting in more polarization of T cells in Th2 phenotype (53). Furthermore, cytokines such as TNF-α released by mast cells, promote dendritic cell

maturation and migration. In addition, mast cell derived TNF-α is involved in the recruitment of neutrophils (54) and activation of macrophages (55), which are important for phagocytosis of particles, dying cells and debris. The recruitment of eosinophils via mast cells is enabled by mast cell released IL-5 and tryptase (56). Moreover, the activation of mast cells is promoted by the production of stem cell factor (SCF) through eosinophils (57). The interaction with the ILCs can be of particular importance in the regulation of airway inflammation caused by epithelial triggering. All 3 subsets are present in the human lung under homeostatic conditions, with ILC2s and ILC3s being the most prevalent (58). It has already been demonstrated that ILCs are in proximity of mast cells in healthy human lungs (59). Until now, studies in humans have mostly focused on ILC2s. A close direct relationship between mast cells and ILCs is suggested: activated mast cells secrete cysLTs and PGD2, which, in turn, are activators of ILC2s (60, 61). Furthermore, mast cells activated in an IgE-dependent manner, are a source of IL-33, that can activate ILC2s (62). Mast cells can activate ILC2s in an indirect way by releasing proteases like chymase and tryptase to cleave IL-33 in a more bioactive isoform (63). In turn, IL-33 on itself can also activate mast cells to produce several activating cytokines. Besides the known role of mast cells in type 2 inflammation, mast cells could also play a role in neutrophilic inflammation and/or ILC3 activation. Studies on airway ILC3s are scarce, however, a functional role for ILC3s in neutrophilic asthma phenotype has been described (13), illustrating the need to better understand the interaction between mast cells and ILC3s.

Considering the adaptive immune system, mast cell expresses both major histocompatibility complex (MCH) I and II molecules, enabling interaction with T cells (38). This interaction results in antigen-specific expansion of T cells. Interacting with CD4+ T cells, producing TNF-α, mast cells augment their own activation, proliferation and cytokine secretion. CD8+ cells are recruited via CCL5 and LTB4 produced by mast cells. In contrast, regulatory T cells (Tregs) are known to have a suppressive role on mast cells, namely by producing inhibitory cytokines. Indeed, IL-10 and TGF-β secreted by Tregs have been shown to inhibit FcεRI by mast cells (64). Furthermore, it was demonstrated that IL-2 produced by IL-33 stimulated mast cells in turn promotes expansion of Tregs, reducing airway eosinophilia (65). IL-4 and IL-13 released from mast cells have the potential to regulate IgE production by B cells. As mast cells express the CD40 ligand, they are able to make cell-cell contact required for IgE production.

Finally, the airway epithelium is described to interact with mast cells. TSLP released from airway epithelium induces the release of mast cell cytokines and when combined with IL-33 an increase in tryptase, carboxypeptidase and cysLT was observed (66). Also activated mast cells may influence epithelium. For example, tryptase increases the release of IL-8 from the epithelium (67).

Mast cells in asthma

Many studies have demonstrated increased numbers of mast cells, as well as, mast cell mediators in bronchoalveolar lavage fluid (BAL) in patients with asthma compared to healthy controls. Using electron microscopy, mast cell degranulation within the airway epithelium and smooth muscles was demonstrated (68, 69). Similarly, increased numbers of mast cells were observed in both epithelium and airway smooth muscles of patients with asthma compared to controls (70, 71). Also gene expressing profiling studies show differentially expressed genes (e.g. TPSAB1 and CPA3) related to mast cells in patients with asthma, especially in asthma patients without ICS, compared to healthy controls (72). Mast cell mediators such as histamine, PGD2 and LTC4 are potent agonists for smooth muscle contraction. Moreover, there exists an inverse correlation between the number of mast cells and airway hyperresponsiveness in patients with asthma (71). Elevated circulating mast cell progenitors are correlated with reduced lung function in patients with allergic asthma, indicating that reduced lung function may reflect pathological changes due to mast cell infiltration (73). A shift from mast cells in the mucosa to the epithelium was observed in asthma patients with type 2 inflammation and indirect airway hyperresponsiveness, suggesting that intraepithelial mast cells are modulators of inflammation in asthma. This interaction with epithelium is proposed to be mediated via IL-33 signalling (70).

The role of mast cells in airway inflammation in asthma has also been supported by the efficacy of drugs, reducing mast cell activation or targeting released mediators. The clinical efficacy of omalizumab in severe atopic asthma indicates the importance of IgE in airway inflammation. However, it does not cure the disease and is not effective in every patient. Mast cell stabilizing drugs such as sodium cromoglycate are not very effective in preventing the underlying inflammation in asthma. Efficiently mast cell targeting drugs therefore remain a challenge. New drugs in the pipeline mainly focus on FcεRI mediated activation such as Syk inhibitor R112 and kit inhibitor masitinib (74). Less is known of the role of the mast cell in

specific non-allergic asthma. Immunohistochemistry showed increased number of FcεRI$^+$ cells in both allergic and non-allergic asthma patient compared to healthy controls (75), indicating that mast cells are also a potential target in IgE-independent non-allergic asthma.

Mast cell models

To gain more insights into the role of mast cells in non-allergic asthma and their interaction with immune cells involved in non-allergic asthma, *in vitro* mast cell models can help. As mast cells are tissue resident cells, tissue explant of lung tissue are used for mast cell function assessment (76, 77). Since mast cells are present in very low numbers in tissues and the isolation process may disrupt the normal cell phenotype, they are not easily used for *in vitro* analysis. Therefore, alternative methods have been developed to study human mast cell interaction with other immune cells.

Different human cell lines have been developed, such as HMC-1, LAD2, LUVA and ROSA cell line. Nevertheless, they also have their limitations. HMC-1 cells, deriving from mast cell leukaemia cells, only poorly express FcεRI and lack well-formed granules (78). LAD2 cells are derived from a patient with mastocytosis but have a slow doubling time and are considered to be intermediately differentiated mast cells expressing low levels of tryptase and chymase (78). LUVA cells originate from peripheral blood CD34$^+$ cells of a patient with aspirin exacerbated respiratory disease and have a weak release of β-hexosaminidase upon IgE/FcεRI cross-linking (79). The most recent mast cell line is ROSA, cultured from normal cord blood and express functional FcεRI but lack chymase (80).

Alternatively, human progenitor cells can be differentiated into primary mast cells. Human CD34$^+$ progenitors are obtained from bone marrow, cord blood or peripheral blood, of which the last is the most accessible and results in mature mast cells (81). However, these differentiation protocols can take up several weeks and can generate different phenotypes as mast cells are sensitive to their environment (82–84). So, it is important to fully characterize the obtained cells. Generally, SCF and IL-3 are added to promote survival, proliferation and differentiation of *in vitro* cultured mast cells (85). Adding FBS in later weeks of culture period is described to increase viability, while in the beginning it will result in low number of mature mast cells (86). Recently, a promising shorter protocol (3 weeks) to differentiate mast cells was published by Cop *et al.* (87).

In summary, mast cells are complex cells which can be activated in an IgE dependent and IgE-**in**dependent way, making them important in both allergic and non-allergic asthma. Most research focuses on mast cells in allergic disease. More research is necessary to fully understand the role and multiple functions of mast cells in asthma.

2. The exposome

The concept of exposome was introduced by Wild *et al.* (88) who adressed the challenge of measuring environmental exposure in molecular epidemiology. Our respiratory system, due to its anatomical positioning, acts as a sentinel for the environment in which we live (89). On one hand it is equipped to protect our inner body from harmful substances, but on the other hand it is susceptible to damage and development of sustained diseases. There is compelling evidence that allergic airway diseases, such as allergic asthma, are influenced by exposure to allergens (89). In the past two decades, several other asthma phenotypes have emerged and we now have a better understanding of the non-allergic triggers of these diseases (90, 91). Climate change, air pollution and lifestyle factors all have the potential to contribute or modify disease onset, progression and immunological airway response (92). In this part of the thesis, we further focus on the effect of intense physical exercise and air pollution exposure on the airways.

2.1. Exercise

Intense physical exercise in athletes, increases the risk to develop exercise-induced bronchoconstriction (EIB). In particular, ventilation increases from 6 L/min to 100-150 L/min and even beyond 200 L/min in adult elite athletes and switch from nasal to mouth breathing occurs during intense exercise. This might act as a stressor of our respiratory system. Within this thesis, an athlete is defined as a person who is trained in sports that requires physical activity (≥ 12 hours of sport a week). Especially endurance athletes are considered to be at risk to develop EIB. Definition, diagnosis and screening of EIB are reviewed in **chapter 2**.

Pathogenesis

Regarding the underlying mechanism of EIB, different theories are described: thermal hypothesis, osmotic hypothesis, neurogenic inflammation and also the induction of epithelial cell damage, resulting in the release of different inflammatory mediators and bronchoconstriction (93, 94)(figure 5). Airway cooling resulting from conditioning of inspired

air, causing vasoconstriction, and post-exercise rewarming of airways have been proposed as "thermal" mechanism. This results in vascular leakage and oedema which leads to airway narrowing. Airway dehydration as a result of increased ventilation, resulting in augmented osmolarity of the airway-lining fluid and a reduction in volume of the cell, is described as "osmotic" mechanism. Cell shrinkage will result in the release of soluble phospholipase A2 to produce arachidonic acid, which is taken up by lipid bodies in surrounding mast cells and eosinophils (94). The activation of these inflammatory cells induces the release of mediators, such as histamine, cysLTs and PGD2, which leads to airway smooth-muscle contraction and airway oedema. More recently, the nervous system has been proposed as other contributor to EIB (93). Sensory nerves may be directly activated by osmotic stress. Increased circulating levels of substance P have been found after intense exercise (93). In addition, there is evidence that the release of neurokinin A may occur via the cysLT-associated activation of sensory nerves and is correlated with bronchoconstriction (95). Furthermore, acetylcholine released from the epithelium may have stimulant effect upon airway inflammation. The role of TRPA1 in non-allergic bronchial hyperreactivity was demonstrated in a mouse model after single instillation of hypochlorite-ovalbumin, independent of bronchial influx of inflammatory cells (96). Finally, recent evidence suggests that the high ventilation rate causes "epithelial damage", with the release of mediators from epithelial cells as the underlying mechanism (97–99). This damage, which can be more pronounced if additional triggers are present, might lead to uncontrolled airway inflammation, can aggravate the process and raise EIB (100), potentially resulting in persistent asthma. Multiple studies support this mechanism, demonstrating increased serum and urinary levels of club cell protein 16 (CC16), a marker of epithelial damage (93, 101, 102). Furthermore, increased sputum concentrations of DAMPS such as uric acid, HMGB1 are observed (101, 103). Damage of the epithelial barrier could facilitate the passage of inhaled substances and the interaction of those substances with immune cells.

These mechanisms are considered to initiate inflammatory responses seen in airways of elite athletes. Typically, increased number of inflammatory cells are found in airways of high-risk sport disciplines. Mainly neutrophils are found to be increased (99). Moreover, sputum neutrophil count correlated with the number of training hours a week in both swimmers and cold-air athletes (104). Furthermore, pro-inflammatory mediators such as IL-8 and cysLTs, are

found in sputum of high-risk athletes (e.g. marathon runners, swimmers and winter sport athletes) (93, 94, 105). Mediators released by damaged epithelium, can stimulate cells of innate immune system like macrophages, with the induction of pro-inflammatory cytokines (IL-1β, IL-6 or TNF-α) and are suggested to induce Th17 inflammation (93, 106). The severity of EIB was also found to be correlated with increased concentrations of inflammatory mediators such as CysLTs, PGE2, TXB_2, and LTB_4, supporting a potential inflammatory basis for EIB (107). Besides, it is well recognized that mast cells have a role in EIB and release mediators such as histamine, CysLTs, which act on smooth muscle causing airway constriction (108).

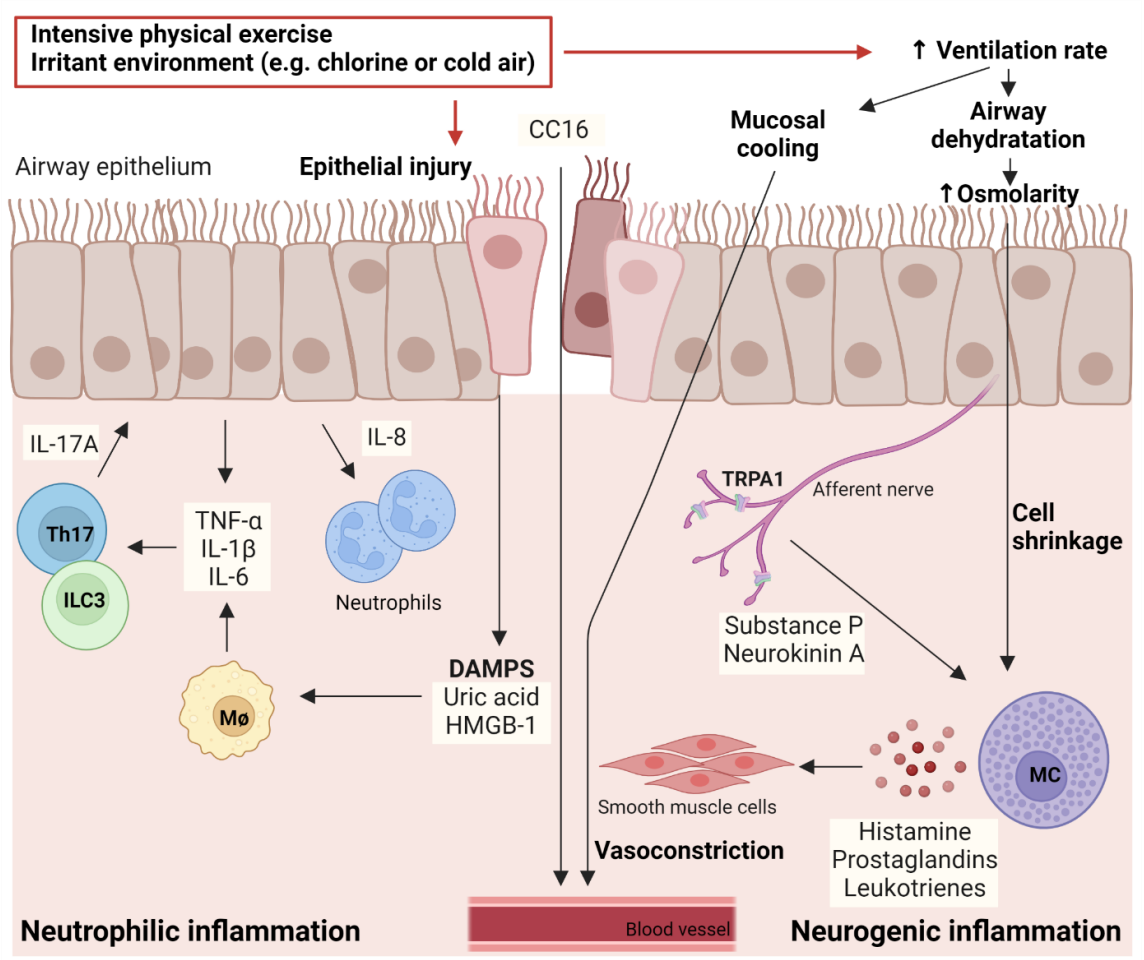

Figure 5: Mechanisms EIB. Adapted from Couto *et al.* 2018. (93)**.** CC16, club cell protein 16; DAMPs, damage-associated molecular patterns; HMGB-1, High-mobility group box 1; ILC3, innate lymphoid cell type 3; MC, mast cell; Mø, macrophage; Th17, Type 17 T helper cell; TRPA1, Transient receptor potential ankyrin 1. (Created with Biorender.com)

2.2. Air pollution

Air pollution and, more specifically particulate matter can deposit in several regions in the lung, inducing oxidative stress and inflammatory response in the airways. Here as well, the airway epithelium is considered to be a key player in the response to this external trigger. An overview of recent literature on the effect of air pollution on the respiratory system with an immunological focus on the effects on healthy and diseased airways can be found in our review, Goossens J *et al.* Atmosphere 2021 (109).

Chapter 2

How to detect young athletes at risk of exercise-induced bronchoconstriction?

REVIEW

Manuscript published in Paediatric Respiratory Reviews. 2022

Janne Goossens[a], Tatjana Decaesteker[b], Anne-Charlotte Jonckheere[a] Sven Seys[a], Sophie Verelst[c], Lieven Dupont[b,d], Dominique MA Bullens[a,c]

[a] KU Leuven, Department of Microbiology, Immunology and Transplantation, Allergy and Clinical Immunology Research Group, Leuven 3000, Belgium
[b] KU Leuven, Department of Chronic Diseases, Metabolism and Ageing, Laboratory of Respiratory Diseases and Thoracic Surgery (BREATHE), Leuven 3000, Belgium
[c] UZ Leuven, Clinical division of Paediatrics, Leuven 3000, Belgium
[d] UZ Leuven, Clinical division of Respiratory Medicine, Leuven 3000, Belgium

1. Abstract

Exercise-induced bronchoconstriction (EIB) is a prevalent condition in elite athletes caused by transient airway narrowing during or after exercise. Young athletes nowadays start early to perform high level exercise, highlighting the need to screen for EIB in a younger population. The purpose of this review was to evaluate current evidence of pre-tests with high probability to predict a positive provocation test in young and adolescent athletes, aged 12-24 years and thus indicate whether a young athlete is at risk of having EIB. Up to now, there is no validated screening test available to increase the pre-test probability of a provocation test of EIB in young and adolescent athletes. We would recommend that a clinical guideline committee might consider the development of a flowchart to screen for EIB in adolescent athletes, composed of a symptom-based questionnaire focusing on wheezing during exercise, atopic state, reversibility test (to exclude EIB with asthma) and completed with markers in blood/serum. However, more research is necessary.

2. Introduction

Physical exercise, even though absolutely beneficial for human well-being, is a well-known trigger to induce bronchoconstriction. Exercise induced bronchoconstriction (EIB) is defined as the transient reversible narrowing of the lower airways that follows physical exercise (110). Typical symptoms include wheeze, dyspnoea, cough, chest tightness and excessive mucus production associated with exercise (111). The terms EIB and exercise induced asthma (EIA) are often used as synonyms. The ATS guidelines defined EIB as an airway contraction associated with exercise (110). A Joint Task Force on Practice Parameters in Allergy and Immunology defined EIB as a transient narrowing of the lower airways following exercise in the presence or absence of clinically recognized asthma and stated that EIA was no longer a preferred term, because it might incorrectly imply that exercise causes rather than triggers an asthma attack (112). Del Giacco *et al* referred to EIB as the bronchoconstrictive response and to EIA when bronchoconstriction is associated with asthma symptoms (113), while more recent publications recommended classifying EIB into 1 of 2 subtypes: EIB with asthma (EIB_a), or EIB without asthma (EIB_{wa}) (93, 94).

Recent data indicates that adult elite athletes have a higher risk for developing EIB (111, 114–116). The prevalence of EIB in elite athletes ranges from 30% to 70%, depending on the type of sport and the environment of exercise (94). Especially endurance athletes exposed to cold air (cross-country skiers) or chlorine (by-)products (swimmers) are at risk (117–119). The elevated risk to develop EIB in athletes is most likely due to increased breathing capacity during intensive exercise, leading to airway dehydration, increased airway osmolarity and/or epithelial cell damage (93, 100). When exercise is performed in potential harmful environmental conditions, such as extreme cold or chlorine by-products the risk to develop EIB increases (99, 120, 121). Furthermore, there is growing evidence that athletes fail to recognize and report symptoms of EIB (122, 123). A study of elite British athletes (24.0 ± 4.1 years) demonstrated a high proportion of previously undiagnosed athletes who were diagnosed with a positive eucapnic voluntary hyperpnea (EVH) test (73%), and suggested to routinely screen for EIB in elite athletes (124). Whether EIB is completely reversible after stopping their elite sports career or continues in persistent asthma, is still a matter of debate (93, 125, 126).

The elevated risk for EIB seen in athletes is typically attributed to the years of intensive training. There is a tendency that athletes start to exercise intensively at an early age. It has been demonstrated that EIB can already be present early on in the sports career or during adolescence. Jonckheere *et al* reported that 24.5% of tested early-career athletes (12 - 13 years old) suffer from EIB (101). This is even slightly higher than the reported prevalence of 15% of EIB in young elite athletes (5 - 18 years old) in the meta-analysis of Aguir *et al* (127). This high prevalence points towards the need to identify athletes who would benefit from screening for EIB. As in adults, a high proportion of young athletes is therefore currently training and performing sports with undiagnosed and/or uncontrolled EIB. The development of EIB may have a deleterious impact on ventilatory capacity and sports performance (128). Altogether, there is an urgent need for screening tools to detect young athletes at risk for EIB even at the start of their sports career. At present, medical screening of athletes focuses on detection of major musculoskeletal and cardiac abnormalities. We propose that sports physicians supervising athletes should also actively screen for respiratory diseases.

It is important to accurately diagnose EIB to reduce its potential impact on respiratory health and sports performance. However, diagnosing EIB is clinically challenging as EIB may be present with nonspecific symptoms. Therefore, it is recommended that diagnosis is based on objective testing, most typically defined as a fall in FEV_1 below a predefined threshold (e.g. 10%) after a provocation test (110). In addition, asthma must first be identified to distinguish EIBa and EIBwa (e.g. by performing reversibility test with β_2-agonist or a bronchial challenge test). Due to the many factors that influence the outcome of an exercise 'field' test, surrogate tests were developed that could be more easily standardized for routinely use in a hospital or laboratory. The International Olympic Committee recommends the EVH challenge, a surrogate for exercise, as bronchial provocation challenge test in athletes (129). The EVH test was adapted to be performed in high school elite athletes (130). It is generally considered as the gold standard diagnostic tool in athletes, although this has been criticised (131). Alternative indirect provocation tests are inhaled hypertonic saline challenge and dry powder mannitol challenge. Both tests were developed after the EVH test as a simplification, since no special gas mixture is required (132). Direct challenges include methacholine and histamine challenge, acting on airway smooth muscle to cause bronchoconstriction. These are thought to be less specific for EIB and more accurate to document hyperresponsiveness in asthma.

The purpose of this review was to evaluate current possibilities to increase pre-test probability in young athletes to identify subjects at risk for EIB.

3. Literature search methodology

We examined available English literature since 1990 in Pubmed, Ovid Medline and Embase on screening tools for EIB in young and adolescent athletes, aged 12-24 years, in which the method for EIB diagnosis was based on provocation testing and not solely on a questionnaire.

4. Screening tests to identify subjects at risk

The gold standard to identify EIB in athletes remains the EVH test. However, performing an EVH test in all elite athletes as yearly screening test is considered to be not cost-effective. Moreover, the necessary equipment and expertise is not available in every center. Hence, we focused in this review on screening tools that may predict a positive EVH or exercise test and thus indicate whether a young athlete is at risk of having EIB. The usefulness of screening tools depends on the prevalence of the condition, the ability to treat the condition, the predictive value of the test, the cost-effectiveness of the test and the safety profile of the test. In order to be implemented into daily clinical practice, the screening test needs validation in terms of reliability and repeatability. In literature several screening tools have been described: questionnaires, a diagnosis of atopy, exhaled nitric oxide (FeNO) and markers in blood or sputum, which are all discussed in next sections.

a. Screening by questionnaires

In 1991, Shield *et al.* suggested to screen high school athletes by means of a questionnaire (133). The advantage of using questionnaires is that they can be applied in a large number of young athletes, don't require a lot of time and are less expensive compared to other screening methods. Questionnaires used as screening tools for EIB particularly focus on history (previously diagnosed asthma/allergy and family history), the presence of symptoms (wheezing, chough, chest tightness, dyspnea or excess mucus production) and medication use. In view of this, significantly more EIB+ athletes were found in symptomatic athletes compared to non-symptomatic athletes (134). Table 1 summarizes the characteristics of studies evaluating the role of questionnaires to diagnose EIB in young and adolescent athletes. Nine studies out of 17 reported an association with EIB risk (table 1A) (135–143). The highest

sensitivity was generally found for the question regarding family history of asthma or allergies. For instance, Feinstein *et al.* reported that a personal history of asthma was a predictive factor for EIA, while self-reporting symptoms did not appear to be sensitive enough to identify athletes with EIA (137). High levels for specificity varying from 85% to 97% were found for questions regarding symptoms of EIB like chest tightness, cough or wheeze associated with exercise. In contrast, self-reported symptoms for EIB have been reported to have a low associated sensitivity varying from 13% to 42%. Based on a linear regression analysis, a history of exercise induced respiratory symptoms was found to be predictive for EIB (136). Especially the experience of wheezing during exercise was significantly associated with a positive EVH test, as reported in 2 studies. A positive correlation was also found between missing school/work due to chest tightness or shortness of breath and EIB diagnosis (143).

On the other hand, other studies failed to demonstrate an association between questionnaires and the objective diagnosis of EIB (119, 121–124, 144–150). This points towards the existence of a population of asymptomatic athletes who show objective evidence of EIB. Burnett *et al.* found that the majority of young athletes with EIB reported no symptoms (151). This may be attributed to a lack of awareness of symptoms suggestive of EIB in children. Athletes may also consider dyspnea or chest tightness as a part of their hard and intensive training. Furthermore, the predictive value of symptoms for EIB is also affected by a high proportion of young athletes reporting respiratory symptoms in the absence of EIB. This highlights the need to consider alternative causes for their symptoms like exercise-induced laryngeal dysfunction or exercise induced hyperventilation (152). On the other hand, respiratory complaints are often also a sign of “exhaustion”.

It is widely accepted that adult athletes sensitized to respiratory allergens have higher rates of EIB than other athletes (116, 153, 154). Whether this is the case in adolescent elite athletes is not completely clear. The AQUA questionnaire is a validated questionnaire to quantify the presence and severity of allergic disease in adult athletes (155). Recent studies also demonstrated the feasibility of performing AQUA in young athletes with specificity of 78% and sensitivity of 62.5% to predict atopy (156, 157). Even at the age of 7-14 years, the paediatric AQUA was a valid and reliable tool for screening of atopy (158). As respiratory symptoms are an important component of the questionnaire, it is suggested that the AQUA questionnaire might be used as a pre-screening tool for assessment of airway health in athletes. A study in adult athletes indicates that a negative AQUA score (<5) offers an excellent diagnostic tool to

rule out EIB (159), while in young football players the AQUA score was not predictive for EIB (136).

Although associations were found between certain symptoms and presence of EIB in several studies, they do not seem to achieve a desirable sensitivity or specificity to identify adolescent athletes at risk for EIB. Symptoms and history alone are not adequate to screen for EIB in this population. However, it is certainly interesting to investigate the cause of symptoms associated with exercise. We suggest that questionnaires can be used as a first step in pre-screening tools for EIB, but using only questionnaires as screening tool will be unreliable.

Table 1*:* Characteristics of included studies in young and adolescent athletes investigating questionnaires in association with EIB.

Source	Age	Cases of EIB/sample size	Provocation test	Questionnaires			Association with EIB
				Medical history	Symptoms	Medication use	
A CHILDREN AND ADOLESCENTS (12 – 24 years)							
Bavarian *et al* (135)	7 - 16 years	74/371	Exercise test	/	x	/	**Sensitivity** and **specificity** of self-reported symptoms for EIB diagnosis were **13%** and **89%,** respectively**.**
Bougault *et al* (136)	20 ± 4 years	7/44	EVH test	x	x	/	Main determinant of EIB according to multiple linear regression analysis was self-reported exercise-induced symptoms (coefficient: 0.80; P = 0.001).
Bougault *et al* (119)	20 ± 4 years	28/89	Metacholine and EVH test	x	x	/	0
Clearie *et al* (121)	11 - 16 years	13/36	Exercise test	x	x	/	0
Feinstein *et al* (137)	14 - 18 years	17/48	Exercise test	x	x	x	Logistic regression analysis identified a personal history of asthma as predictor for EIA (P < 0.05).
Hallstrand *et al* (147)	11 - 17 years	24/256	Exercise test	x	x	/	0
Hammerman *et al* (138)	13 - 18 years	95/801	Exercise test	x	x	x	**Sensitivity and specificity** of the screening questionnaire was **42%** and **95%,** respectively.
Kukafka *et al* (139)	16 ± 1 year	19/214	Exercise test	x	x	/	A history of wheezing (P < 0.001), residence in a poverty area (P < 0.0001), race (P = 0.01) and history of asthma (P < 0.001) were significantly associated with EIB.
Mansournia *et al* (140)	19.3 ± 2.7 years	50/463	Exercise test	x	x	x	**Sensitivity** and **specificity** of self-reported symptoms for

							EIB diagnosis were **27%** and **85%**, respectively.
Molphy *et al* (141)	21.9 ± 3.7 years	18/136	EVH test	x	x	x	Significant association between the experience of wheezing during exercise and EVH result ($P < 0.05$).
Parsons *et al* (123)	17 - 23 years	42/107	EVH test	x	x	/	0
Romberg *et al* (145)	14 - 17 years	14/101	Mannitol and exercise test	x	x	x	0
Rundell *et al* (122)	22 ± 4.4 years	42/158	EVH test	x	x	x	0
Rundell *et al* (149)	20 ± 4.5 years	23/23	Exercise test	/	x	/	0
Rupp *et al* (150) (abstract)	12 - 18 years	22/166	Exercise test	x	x	/	0
Sallaoui *et al* (142)	17 - 23 years	14/107	Exercise test	x	x	x	**Sensitivity** and **specificity** of self-reported symptoms for EIB diagnosis were **36%** and **97%,** respectively.
Thole *et al* (143)	17 - 24 years	16/114	Exercise test	x	x	x	Positive correlation of EIB diagnosis with question: "Have you ever missed work or school because of chest tightness or shortness of breath?"
B ADOLESCENTS (18 – 24 years)							
Burnett *et al* (151)	18 - 23 years	34/80	Exercise test	x	x	x	0
Dickinson *et al* (124)	24.0 ± 4.1 years	78/228	EVH test	x	x	/	0
Parsons *et al* (148)	18 - 23 years	4/144	EVH test	x	x	/	0
Vakali *et al* (144)	20.7 - 22.5 years	10/60	EVH test	x	x	/	0

EIB, exercise-induced bronchoconstriction; EVH, eucapnic voluntary hyperventilation.

b. Screening by diagnosing atopy

An increased prevalence rates of allergic diseases have been reported in athletes (116, 160–163). Zwick *et al.* demonstrated that competitive swimming may facilitate sensitization to air-borne allergens due to epithelial damage induced by the frequent exposure to chlorine and chlorine by-products in swimming pools (164). Atopy is particularly frequent in elite athletes. At the Olympics in Sydney, 41% of the Australian athletes had a positive skin tests to at least one inhalant allergen (163). In another study investigating the atopic state in endurance athletes, the prevalence of atopy was even 57.6% (165). Atopic state can be verified by serological tests or by skin-prick test to detect antigen-specific IgE antibodies to

environmental allergens; either to series of common allergens or to self-reported allergens based on personal complaints (166).

Table 2 summarizes the characteristics of 6 included studies investigating atopy in association to diagnosed EIB in adolescent athletes. Overall, studies demonstrated a high prevalence of atopy in young athletes (101, 119, 136, 144, 145, 167). Atopic Tunisian athletes presented a higher risk of developing EIB than non-atopic athletes (167). Furthermore, sensitisation to at least 5 allergens was found to predictive for EIB in young athletes (136). Despite the high prevalence of atopy in adolescent athletes, no association of atopy and EIB was demonstrated in other studies (101, 119, 144, 145). To summarize, diagnosing atopy alone is not considered to be sufficient as screening tool for EIB in adolescent athletes. However, when developing a screening test, we need to take in consideration that EIB may also arise from the presence of allergy which lowers the threshold to provoke EIB, especially in the subtype of EIB with asthma.

Table 2: Characteristics of included studies in young and adolescent athletes investigating atopic state in association with EIB. The prevalence of atopy in the study population is reported.

Source	Age	Cases of EIB/Sample size	Provocation test	Sport discipline	Prevalence	Association to EIB
A CHILDREN AND ADOLESCENTS (12 – 24 years)						
Bougault *et al* (136)	20 ± 4 years	7/44	EVH test	soccer players	49%	Sensitisation to at least 5 allergens
Bougault *et al* (119)	20 ± 4 years	28/90	Metacholine and EVH test	swimmers and winter sport athletes	74%	/
Jonckheere *et al* (101)	12 - 13 years	29/117	EVH test	Mixed sport disciplines	33%	/
Romberg *et al* (145)	13 - 24 years	14/97	Mannitol and exercise test	swimmers	66.7%	/
B ADOLESCENTS (18 – 24 years)						
Sallaoui *et al* (167)	20.8 ± 2.7 years	32/326	Exercise test	mixed sport disciplines	26.9%	Higher frequency atopy in EIB+ athletes
Vakali *et al* (144)	20.7 – 22.5 years	36/111	EVH test	mixed sport disciplines	48.7%	/

EIB, exercise-induced bronchoconstriction; EVH, eucapnic voluntary hyperpnea

c. Screening by FeNO

Measuring FeNO levels is a quantitative, non-invasive and simple way to study the degree of eosinophilic airway inflammation. FeNO measurements are used to identify uncontrolled asthma and as predictor of the response to corticosteroids (168). NO is produced by inducible nitric oxide synthase in airway epithelial cells. Although several studies have tried to define normative FeNO levels, clear cut-off points were not established (169). This can be explained by the fact that FeNO levels are influenced by several parameters like age, height, smoking

status, intake of arginine containing foods, prior spirometry and airway infections (170). As FeNO is associated with eosinophilic asthma, and as swimmers show often a more neutrophilic airway inflammation pattern (99), the role of FeNO as predictor for EIB in this population may seem questionable. However at least in adult athletes, FeNO levels >50 ppb provided a good specificity for a positive EVH test (171, 172).

Table 3 summarizes the characteristics of 7 included studies investigating FeNO levels in association with EIB status in adolescent athletes. Only one abstract reported significantly elevated FeNO levels in EIB+ athletes compared to EIB- athletes (17.0 ppb versus 13.0 ppb respectively) (173). Having high FeNO levels (>20 ppb) was an independent predictor for EIB (173). A similar cut-off (>22 ppb) was identified in athletes in a separate cohort aged 14 - 31 years by Voutilainen *et al.* (174). However, the sensitivity (55%) and the specificity (71%) were poor. Other studies in adolescent athletes with FeNO did not report higher levels in EIB+ subjects (121, 145, 148, 175–178). In contrast, a study in asthmatic children showed that their baseline FeNO levels were significantly increased and correlated with the magnitude of the maximal decrease in FEV_1 after exercise (179).

FeNO levels do not seem to be associated with EIB in young athletes and are consequently considered to be a poor pre-screening test in young athletes. Although, it is suggested that FeNO may help to distinguish EIB with asthma and EIB without asthma in athletes (175).

Table 3: Characteristics of included studies in young and adolescent athletes investigating FeNO levels in association with EIB.

Source	Age	Cases of EIB/Sample size	Provocation test	FeNO EIB+	FeNO EIB-	P-value	Cut-off
A CHILDREN AND ADOLESCENTS (12 – 24 years)							
Clearie *et al* (121)	11 - 21 years	13/36	Exercise test	16.2 ppb	25.1 ppb	P > 0.05	/
Ersson *et al* (177) (abstract)	13 - 17 years	24/98	Exercise test	/	/	P > 0.05	/
Johansson *et al* (173) (abstract)	13 - 16 years	39/128	Exercise test	17.0 ppb	13.0 ppb	P = 0.03	> 20 ppb
Millward *et al* (175)	17 - 23 years	16/74	EVH test	18.7 ppb	21.5 ppb	P > 0.05	/
Pedersen *et al* (176)	14.3 ± 1.2 years	8/33	Metacholine and EVH test	18.8 ppb	17.3 ppb	P > 0.05	/
Romberg *et al* (145)	13 - 24 years	14/101	Mannitol and exercise test	/	/	/	> 20 ppb
B ADOLESCENTS (18 – 24 years)							
Parsons *et al* (148)	18 - 23 years	4/144	EVH test	13.3 ppb	24.4 ppb	P > 0.05	/

EIB, exercise-induced bronchoconstriction; EVH, eucapnic voluntary hyperpnea; FeNO, fractional exhaled nitric oxide

d. Screening by markers in blood

Few studies have investigated the potential role of serum markers as screening test for EIB in young and adolescent athletes. Serum NO and plasma endothelin-1 levels, reflecting airway inflammation, were found to be significantly elevated in young soccer players with EIB after exercise compared to healthy controls (180). In addition, Jonckheere *et al* demonstrated a significant correlation between serum club cell protein 16 (CC16) levels and the maximal fall in FEV_1 after EVH test (101). It has been demonstrated that acute exercise induces increased levels of CC16 (93), while the use of CC16 levels to identify athletes at risk of EIB, is not completely clear. Another biomarker, serum uric acid, was shown to be higher in EIB+ athletes compared to EIB- athletes (101). CC16 and uric acid are both markers of epithelial damage (93). Further validation studies are needed to determine whether serum or blood biomarkers are suitable candidates to complete the pre-screening tool for EIB in young and adolescent athletes.

e. Screening by sputum induction

Markers of inflammation or epithelial damage in sputum are the most reliable non-invasive reflection of the airways (181). Induced sputum has been commonly used for the analysis of differential cell counts, soluble markers in sputum supernatant and cytokine expression of sputum cells of patients with asthma (182, 183). Limiting factors of the use of sputum samples as biomarkers are the need of trained experienced personnel and the availability of adequate equipment and facilities. In addition, it is a time-consuming technique and it is challenging to obtain high quality samples. It has been argued that intensive endurance training leads to an increase in sputum neutrophils (93). An alternative tool to identify the cellular sputum profile is to use sputum or serum calprotectin levels, which is released upon neutrophil activation. However, calprotectin correlated only with neutrophil percentage in asthmatic patients and not in a small group of athletes, suggesting a minor role for neutrophils as screening tool in athletes (184). Few studies reported inflammatory markers in sputum in non-asthmatic athletes. It was demonstrated that young athletes with sputum IL-1β mRNA levels higher than 300 are more prone to have or develop EIB ($P = 0.0331$, PPV = 0.85) (101). In addition it was demonstrated that athletes with sputum IL-8 mRNA levels lower than 190 are at very low risk of developing EIB ($P = 0.003$) (101). Concentrations of inflammatory mediators (Cys-leu, PGE2, histamine, TXB2 and LTB4) are significantly elevated in EIB+ athletes compared to EIB- athletes. Furthermore, the severity of EIB was significantly correlated with increased

concentrations of select inflammatory mediators (P = 0.0025) (107). Nevertheless, an optimized and easy to use method for sputum analysis needs to be developed to consider markers in sputum as a screening tool to identify athletes at risk for EIB. Practically, a specific set of biomarkers should be developed, representing a 'fingerprint' of EIB.

5. Discussion

Screening tools should be applied routinely to identify young athletes at risk for EIB, who can then be referred for an EVH or exercise test. The introduction of a screening policy for EIB would improve the management adolescent athletes. Preliminary screening tests could point out athletes at risk for EIB at a low cost. Additionally, proper medical screening can maximize an athlete's participation and function in a sport. Accordingly, it will improve diagnosis of EIB and EIB control. Furthermore, a screening tool for EIB should also allow physicians to rule out vocal cord dysfunction (VCD), a frequently overlooked differential diagnosis of EIB. The optimal screening tool should be an inexpensive and non-invasive test with a high level of sensitivity and specificity to detect EIB (185).

Many studies support the need for screening EIB in elite athletes. On the other hand, routinely screening of all athletes may be an ineffective strategy as the prevalence of EIB varies widely between groups of athletes. It seems more appropriate to use a screening test to specific subpopulations who perform activities with a high risk to develop EIB, like swimming, cycling, cross country skiing and other high-altitude sports. To our knowledge, no literature has studied at what age such screening should performed. Although physical mobilization of adolescents is of utmost importance to prevent asthma, too much stress on the airways results in epithelial damage, chronic inflammation and associated bronchoconstriction. This negative impact of intensive exercise on the developing airways in adolescents must be detected and avoided. When screening for EIB in young athletes, we would also suggest to consider the number of hours of sport a week, preferentially testing those exceeding 10 hours of sport a week.

More research is needed to develop an effective screening tool for EIB. Firstly, few studies are performed on the relationship of different risk factors for EIB in young athletes. Secondly, most studies are descriptive and not investigating predictive characteristics or ROC analyses. Finally, the variable prevalence of EIB in different studies in combination with multiple potential provocation methods for diagnosing EIB further complicate the development of a

universal screening tool for EIB. In literature, various terms are used to describe EIB and EIB may be present with or without underlying asthma.

6. Conclusion

Up to now, there is no validated screening test available to predict a positive provocation test of EIB in adolescent athletes, indicating young and adolescent athletes at risk for EIB. Current literature demonstrated the use of no single tool, neither questionnaires, nor diagnosis of atopy, nor FeNO, nor biomarkers of airway inflammation, is adequate to screen for EIB in adolescent athletes. We suggest new studies on screening tools for EIB to focus on a step wise approach combining the discussed tools. Based on this, we strongly suggest that a clinical guideline committee might consider the development of a screening flowchart in 12-24 years old athletes to identify athletes at risk for EIB, for whom an EVH test is recommended. This flow diagram should best contain the following steps: 1) Ask for respiratory symptoms after/during exercise 2) perform AQUA and/or skin prick test, 3) perform reversibility test to rule out EIBa, 4) exclude VCD and 5) search for biomarkers; but its sequence is still a matter of debate (figure 1). Additional questions arise about who of the young elite athletes would benefit from such screening test considering age, sport discipline and the number of hours of sport a week.

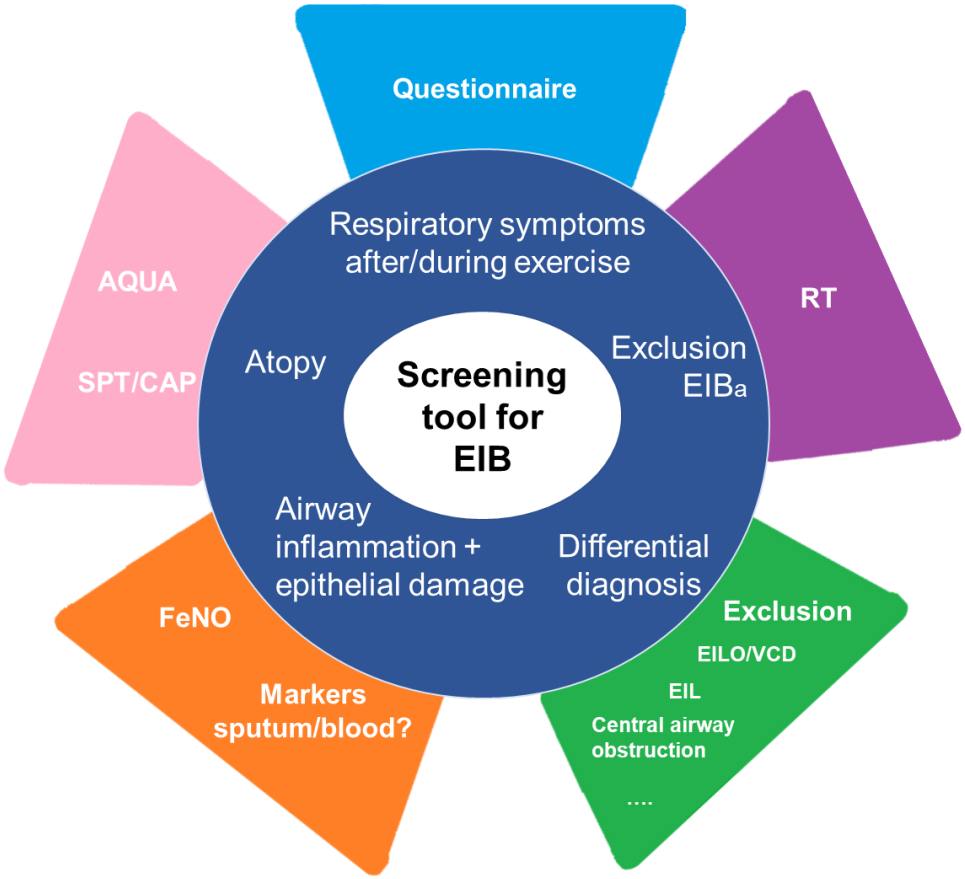

Figure 1: Key elements for development flow diagram to screen for exercise-induced bronchoconstriction (EIB) in young and adolescent athletes. This screening tool will increase the pre-test probability of a positive EVH test, by identifying athletes with a high risk for EIB. AQUA, Allergy Questionnaire for Athletes; CAP, ImmunoCAP; EIB, Exercise-induced bronchoconstriction; EIBa, Exercise-induced bronchoconstriction with asthma; EIL, Exercise-induced laryngomalacia EILO; Exercise-induced laryngeal obstruction; FeNO, fractional exhaled nitric oxide; RT, reversibility test; SPT, skin prick test; VCD, vocal cord dysfunction

Chapter 3

Rationale and aims

Via inhalation, humans are continuously exposed to external triggers in the air, inducing epithelial triggering and potential damage. This epithelial damage may induce the activation of an immunological reaction, which is essential to return to tissue homeostasis. Persistent activation of immune cells may evolve into dysregulation of normal healing responses, causing a possible start of chronic inflammation. We hypothesize that epithelial triggering by cytokine release directly activates cells in close contact with airway epithelium such as ILCs and mast cells. In this context, mast cells might be activated in an IgE-dependent way, which is best studied in the context of allergic asthma, but also in an IgE-independent manner. Indeed, mast cells also express a variety of receptors enabling mast cell IgE-independent activation by diverse molecules (e.g. lipids, chemokines, adenosine, neuropeptides). Furthermore, we hypothesize that the newly identified mast cell receptor MAS-related G protein-coupled receptor X2 (MRGPRX2) may play an important role in IgE-independent mast cell activation in non-allergic asthma. Activation of this immune cascade, can induce the production and release of various inflammatory mediators inducing bronchoconstriction in a non-IgE mediated way.

In this thesis, we aim to study airway inflammation as a result of external triggers inducing epithelial damage. As athletes, who are in this thesis defined as persons who are trained in sports that requires physical activity (≥ 12 hours of sport a week), have an increased ventilation rate, they are hypothesised to be exposed to more external triggers. This may result in increased epithelial damage and bronchoconstriction. It is known that elite athletes have a higher risk to develop exercise-induced bronchoconstriction (EIB). Specifically, we focus on EIB in young athletes, whose airways are still growing**.** First of all**,** we aim to study EIB in young athletes, investigating specific risk factors such as atopic state. This can contribute to the development of a screening tool to predict young athletes at risk to develop EIB. As such, we hypothesize that screening will improve the management of adolescent athletes and can maximize an athlete's participation and function in a sport (see also chapter 2). Secondly**,** we aim to study in depth potential epithelial cell stimulation after exposure to intense exercise and environmental triggers such as air pollution. We hypothesize that risk of EIB may be elevated by a continuous increase in the intensity and frequency of physical. Additional exposure to environmental triggers such as air pollution could enhance this reaction. The activation of the immune system may induce airway hyperreactivity and inflammation even in healthy subjects and may aggravate these features in asthmatic individuals. Therefore, we

also aim to study the contribution of external triggers to airway inflammation seen in a cohort of adult patients with asthma. Thirdly**,** we aim to study the central role of the mast cell in airway inflammation due to epithelial cell damage with local activation of mast cells and subsequent cellular recruiting. We hypothesize that epithelial cells can directly and/or indirectly (e.g. by neuro-mediators) activate mast cells, also in non-allergic asthma.

AIM 1: Investigate atopy and EIB in young athletes.

a) *Investigate accuracy of AQUA© and FeNO to predict atopy in young elite athletes*

Atopy has been significantly associated with bronchial hyperreactivity and EIB in elite athletes. A screening tool for atopy may help with the early identification of atopy and allergy symptom development, especially of interest to young elite athletes. These young elite athletes include persons (12-18 years) who are trained in sports that requires physical activity (≥ 12 hours of sport a week) and are affiliated to an 'Elite Sports' School'. In **chapter 4,** we investigated the ability of AQUA© and FeNO to predict atopy in a young elite athlete population (n=90).

b) *Study EIB and associated risk factor in adolescent athletes*

Next, we studied EIB in a population of recreational adolescent athletes (12-18 years, n=327) who also perform intense physical exercise (≥ 12 hours/week), but not need to be affiliated to an 'Elite Sports' School', in **chapter 5.** To better identify subjects at risk for EIB we investigated associated factors for a positive EVH test.

AIM 2: In depth analysis of exposure to external triggers on the airways.

a) *Investigate effect of intense exercise and exposure to air pollution in elite athletes*

Participation in high-intensity exercise in early life might act as stressor to the airway barrier. In **chapter 6** we investigated the effect of intense exercise and environmental exposure to air pollution on airways of young elite athletes (n=90) during their early-career compared to healthy controls (n=25). Furthermore, we studied the underlying mechanisms of EIB in these young athletes. We hypothesized that next-generation sequencing (RNA-Seq) enables high-throughput and detailed characterization of gene expression levels at the tissue level, unravelling these objectives.

b) *Investigate airway damage based on exposure in asthmatic patients*

It is described that the exposome may alter immunological airway responses. In **chapter 7**, we assessed the effect of environmental exposures such as smoking and presence of work-related exposures to cleaning products, which were retrospectively analysed, on the transcriptome in sputum samples of asthmatic patients (n=103).

AIM 3: Study the involvement of mast cells in non-allergic asthma

a) *Explore mast cells in human sputum samples – pilot study*

As we want to study the central role of the mast cell in non-allergic asthma, a first step is to study the involvement of the mast cell in asthma inflammatory phenotypes. In a pilot study, we identified and characterized mast cells in the airways of subjects with specific non-allergic asthma phenotypes (n=5), patients with allergic asthma (n=5) and healthy control subjects (n=5) (**chapter 8**).

b) *Study human mast cell in vitro*

Furthermore, mast cells might also be activated via direct and/or indirect epithelial damage. Therefore, we need an optimized protocol for human mast cells expressing the MRGPRX2 receptor. In **chapter 9**, we optimized an *in vitro* mast cell differentiation protocol to obtain functional MRGPRX2 expressing mast cells.

Chapter 4

Can AQUA© questionnaire and FeNO predict atopy in early-career athletes?

RESEARCH ARTICLE

Manuscript published in Pediatric Allergy and Immunology. 2023

Janne Goossens[1], Josefien Vandekerckhove[1,2], Anne-Charlotte Jonckheere[1], Ellen Dilissen[1], Sven F Seys[1], Vincent Vanbelle[3], Sven Aertgeerts[4], Jeroen Stappers[5], Koen Peers[6], Marc Raes[2,7], Sophie Verelst[2,7], Jasmine Leus[2,8], Lieven J. Dupont[9,10], Dominique M. A. Bullens[1,2]

[1] Allergy and Clinical Immunology Research Group, KU Leuven, Leuven, Belgium
[2] Clinical division of Paediatrics, UZ Leuven, Leuven, Belgium
[3] Flemish Swimming Federation, Merelbeke, Belgium
[4] Academic Centre for General Practitioners, KU Leuven, Leuven, Belgium
[5] Sports doctor's practice, Wilrijk, Belgium
[6] Sport Medical Advice Centre, UZ Leuven, Leuven, Belgium
[7] Pediatrics, Jessa Hospital Hasselt, Hasselt, Belgium
[8] Pediatric Allergy, AZ Maria Middelares Gent, Gent, Belgium
[9] Laboratory of Respiratory Diseases and Thoracic Surgery (BREATHE), KU Leuven, Leuven, Belgium
[10] Clinical division of Respiratory Medicine, UZ Leuven, Leuven, Belgium

1. Introduction

To the editor,

Allergic sensitization and allergic diseases have been reported to have a high prevalence in athletes (186, 187). Moreover, atopy has been significantly associated with bronchial hyperreactivity and exercise-induced bronchoconstriction in elite athletes (188, 189). Sensitisation to allergens is generally determined by a skin prick test (SPT) and/or allergen-specific serum IgE analysis (190). Recently, the Allergy Questionnaire for Athletes (AQUA©)(155), a screening tool to predict atopy in elite adult athletes, was validated for screening of atopy in young elite athletes (156). This screening tool may help with the early identification of atopy, which may burden the physical performances, and is especially helpful in young elite athletes (190). Increased fractional exhaled NO (FeNO) levels are, considered to be an effective, non-invasive tool to assess the presence of eosinophilic airway inflammation and as tool to diagnose childhood asthma (191–193). This has also been correlated with allergic sensitization (194). Therefore, we hypothesize that measuring FeNO levels may also be a useful non-invasive method to predict atopy in this population. The aim of this study was to investigate the diagnostic accuracy of AQUA© and FeNO in predicting atopy in a young elite athlete population.

2. Methods and results

Therefore, we recruited 90 elite early-career athletes (12-18 years, mean age 15 ± 1.4 years, 57% male) in different sport disciplines attending "Flemish Elite Sports' Schools": basketball (n=24), soccer (n=38), volleyball (n=14) and swimming (n=14). The study was approved by the institutional review board of UZ/KU Leuven (s59778). Subject characteristics are summarized in Table 1. To objectively assess their atopic state, SPT for 9 common aero-allergens: grass pollen, weed pollen, birch pollen, mixed tree pollen (hazel, birch, and alder), house dust mite (HDM, *Dermatophagoides pteronyssinus*), cat, dog, *Alternaria alternata,* and *Aspergillus fumigatus*, together with a histamine control (10 mg/mL) and a negative control were performed (HAL Allergy, The Netherlands). A subject was considered atopic if at least one SPT for an allergen was positive by skin prick test (wheal ≥ 3 mm and larger than the negative control). The total AQUA© score was determined by scoring the questions related to allergy

(Q4 to Q13 as well as Q15), as reported by Jonckheere *et al*. (156). In addition, FeNO levels were measured with Niox Vero (Accuramed, Belgium) recorded in parts per billion (ppb).

Table 1: Subject characteristics

		Sport disciplines				
	Athletes	**Basketball**	**Soccer**	**Volleyball**	**Swimming**	**P-value***
Number (n=)	90	24	38	14	14	
Age (yr)	15 ± 1.4	15.88 ± 1.30	15.39 ± 1.22	16.64 ± 1.08	14.21 ± 1.31	
Gender (M/F)	51/39	20/4	18/20	4/10	9/5	
Atopy (n=)	33 (37%)	10 (42%)	14 (37%)	7 (50%)	2 (14%)	0.2260
Mono-sensitized	8 (24%)	1 (10%)	5 (36%)	2 (29%)	0	
Poly-sensitized (≥2)	25 (76%	9 (90%)	9 (64%)	5 (71%)	2 (100%)	

*P- value was determined via chi- square test amongst different sport disciplines.

Thirty-three of 90 early-career athletes (37%) were atopic, of which 25 (76%) were poly-sensitized (Table 1). The most common positive SPT was observed for grass pollen (n=24), followed by HDM (n=22)(Online repository table E1). AQUA© scores were significantly elevated in atopic athletes (median: 10, P25-75: 3.5-17.5) compared to non-atopic athletes (median: 4, P25-P75: 2-11) (p=0.0084, Figure 1A). Moreover, AQUA© scores were able to predict atopic state with AUC of 0.665 (p<0.0001, Figure 1C). As previously reported in young athletes (156), a cut-off value of 6 had the highest sensitivity (73%) and specificity (53%) (p=0.03, Table 2). Using the traditional cut-off value of 5 yielded a similar sensitivity of 73%, with a slightly lower specificity (51%) (p<0.05, Table 2). Interestingly, FeNO levels were significantly elevated in atopic athletes (median: 22ppb, P25-P75: 13-44 ppb) compared to non-atopic athletes (median: 12ppb, P25-P75: 9 –18 ppb) (p<0.0001, Figure 1A). The AUC for FeNO to predict atopy was 0.765 (p<0.0001, Figure 1C). Athletes with FeNO levels higher than 15 ppb had a significantly higher risk to be atopic, with a sensitivity of 70% and specificity of 65% (p=0.002, Table 2). Using a cut-off value of 20 ppb reduced sensitivity (58%), but increased the specificity to 77% (p<0.001, Table 2). Moreover, AQUA© scores and FeNO levels both significantly correlated positively with the number of positive SPT (Spearman, r=0.31 p=0.0028; r=0.47, p<0.0001 respectively). This gradient was clearly observed for FeNO levels, since FeNO levels of atopic athletes with 2 or more positive reactions were significantly increased compared to non-atopic athletes (Figure 1B). The AUC of FeNO increased from 0.765 (p<0.0001) to 0.814 (p<0.0001) if we considered multiple sensitisations (participants with ≥2

positive SPTs) (Figure 1C), suggesting that FeNO levels might be a useful indicator for multiple sensitisations within atopic athletes. Accordingly, the sensitivity of FeNO ≥15 ppb increased to 80% with same specificity (65%) to detect multiple sensitisations within athletes (Table 2). For FeNO ≥20 ppb, sensitivity decreases to 68%, with specificity of 79%, respectively. Finally, combining FeNO levels (≥15 ppb) and AQUA© scores (≥6), slightly decreased sensitivity (52%), but increased specificity to 82% (p=0.002, Table 2) to detect atopy.

Table 2: Diagnostic accuracy of AQUA© and FeNO for atopy

AQUA score	**Atopic (n=33)**	**Non-atopic (n=57)**	**P-value**	**Sensitivity (%)**	**Specificity (%)**
≥ 5	24	28	0.0455	73	51
< 5	9	29			
≥ 6	24	27	0.0271	73	53
< 6	9	30			
FeNO (ppb)	**Atopic (n=33)**	**Non-atopic (n=57)**	**P-value**	**Sensitivity (%)**	**Specificity (%)**
≥ 15	23	20	0.0021	70	65
< 15	10	37			
≥ 20	19	13	0.0013	58	77
< 20	14	44			
≥ 25	14	5	0.0003	42	91
< 25	19	52			
FeNO (ppb)	**Atopic (≥ 2 sensitisations, n=25)**	**Non-atopic and atopic (1 sensitisation, n=65)**	**P-value**	**Sensitivity (%)**	**Specificity (%)**
≥ 15	20	23	0.0002	80	64
< 15	5	42			
≥ 20	17	15	0.0001	68	77
< 20	8	50			
≥ 25	13	6	<0.0001	52	91
< 25	12	59			
AQUA score and FeNO (ppb)	**Atopic (n=33)**	**Non-atopic (n=57)**	**P-value**	**Sensitivity (%)**	**Specificity (%)**
AQUA ≥6 AND FeNO ≥15	17	10	0.0016	52	82
AQUA <6 and/or FeNO <15	16	47			

P- value, specificity, and sensitivity were determined via a Fisher's exact two-tailed test.

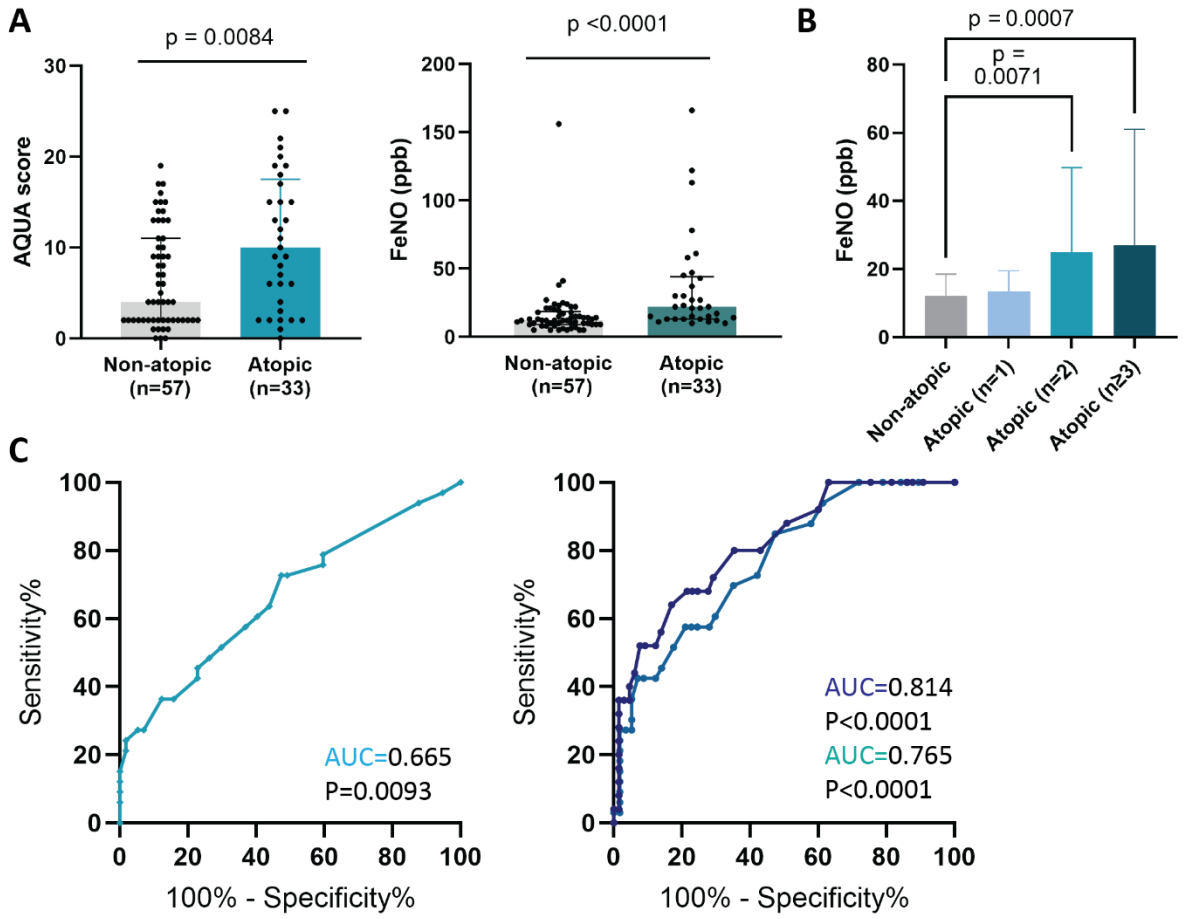

Figure 1: AQUA© and FeNO in young elite athletes. A) AQUA© score and FeNO levels in non-atopic (n=57) and atopic athletes (n=33). **B)** FeNO levels in non-atopic athletes (n=57), atopic athletes with 1 sensitisation (n=8), atopic athletes with 2 sensitisations (n=10) and 3 or more sensitisations (n=15). **C)** ROC Curve indicating the sensitivity and specificity of AQUA© (left) and FeNO (right) to predict atopy and multiple sensitisations (for FeNO). Data are represented as median with interquartile rage in A and B. Mann-Whitney test was used in A to determine the P-value and Kruskal-Wallis test with Dunn's correction for multiple comparisons in B.

3. Discussion

In this study we found a presence of atopy of 37%, which is slightly higher than reported for the general Belgian population (19%)(195), but in line with other authors studying atopy in early-career elite athletes (136, 156). A previous study in Taiwanese children already showed that FeNO levels can discriminate children with and without allergic sensitisations with an optimal cut-off of 15.4 ppb (196). Similarly, we now confirmed in early-career elite athletes the cut-off value of 15 ppb. We are aware of the limitation that we had a limited cohort consisting of 90 athletes only. However, Belgium (Flanders) only has a limited number of young athletes performing at elite level in the respective sport disciplines, who almost all participated in this study. In contrast, Romero *et al.* found that FeNO had limited accuracy to identify atopy in children, but may be a useful indicator of atopy amongst asthmatics with

optimal cut-off of 20 ppb (197). The usefulness of (spirometry-adjusted) FeNO levels to diagnose asthma in children has been demonstrated (192, 193). Based on a questionnaire, six athletes mentioned to have an asthma diagnosis in our cohort. When we excluded those athletes from the analysis, FeNO ≥15 ppb obtained still 68% sensitivity and 64% specificity to predict atopy (p= 0.01) and 81% sensitivity and 65% specificity (p= 0.0003) to predict multiple sensitisations, respectively. Specifically, five of the asthmatic athletes were atopic and had elevated FeNO levels (≥15 ppb), suggesting to have the early-onset clinical endotype of Type-2 asthma seen in athletes (192). While one asthmatic swimmer was non-atopic and also had a lower FeNO level (10 ppb), pointing towards non-Type 2 asthma in athletes (192). We hypothesize that elevated FeNO levels in early-career athletes might point to Type 2 airway inflammation, as typical for allergic diseases but also early onset asthma. We cannot exclude that in subjects with increased FeNO underlying mild asthma might be starting or present. In case of increased FeNO values, specialist referral to exclude potential asthma should be considered (193). Looking at the discrepancy between AQUA© score and FeNO levels we found in athletes with elevated FeNO levels (≥15 ppb) but low AQUA© (<6) scores (n=16), that all athletes with positive SPT (n=6) were sensitized to grass pollen with at least one additional allergen. We hypothesize that these sensitized athletes might not experience a lot of associated symptoms, resulting in a lower AQUA© score. While in the group of athletes with elevated AQUA© scored (≥6) but low FeNO levels (<15 ppb)(n=24), all athletes with positive SPT (n=5) were sensitized to HDM. The elevated AQUA© in athletes with negative SPT was found to be associated in that group by a positive answer to Q4, concerning the diagnosis of allergic disease (e.g. allergy to medication or insect venom) or Q5, concerning suspect of allergic disease independently from any medical diagnosis, both immediately scored as 4 points. Different factors, including sex, height, weight, age, and cigarette smoking may cause variation in FeNO levels. We did not observe a significant difference between male and female athletes (p=0.0683). However, a significant correlation with height (r=0.222, p=0.0354), weight (r=0.245, p=0.0197) and age (r=0.2766, p=0.0083) was noted. Log transformed FeNO levels were found to be a significant predictor of atopy performing binary logistic regression analysis (with ENTER method), considering height (p=0.765), weight (p=0.413), age (p=0.266) and LogFeNO (p<0.001). As atopy is frequently unrecognized and underreported in athletes, the quickly collectable results by AQUA© and FeNO are relevant in the field of intense training in adolescents both for screening and follow-up. Further research may focus on the use of

AQUA© and FeNO in screening for atopic associated conditions, such as bronchial hyperreactivity and exercise-induced bronchoconstriction.

In conclusion, both AQUA© questionnaire and FeNO levels can predict atopy in early-career elite athletes. Our results confirm that in younger age groups an AQUA© score ≥6 is only slightly better than ≥5 to predict atopy in early-career elite athletes. In addition, we demonstrate that FeNO levels are significantly elevated in atopic early-career elite athletes and FeNO ≥15 ppb might be a useful indicator of atopic phenotypes amongst them with higher sensitivity to predict multiple sensitisations. If this finding is further validated, FeNO may provide a simple, real-time non-invasive screening test for atopy among athletes.

4. Supplementary

Table E1: Prevalence of different sensitizations

		Sport disciplines					
	Athletes	**Basketball**	**Soccer**	**Volleyball**	**Swimming**		
Atopy (n=)	33	10 (42%)	14 (37%)	7 (50%)	2 (14%)	**% of all subjects**	**% of all atopic subjects**
Mono-sensitized	8	1	5	2	0		
Poly-sensitized	25	9	9	5	2		
Grass pollen	25	9	10	5	1	28%	76%
Tree pollen	13	6	3	3	1	14%	39%
Weed pollen	6	4	0	2	0	10%	18%
Birch pollen	12	6	3	3	0	13%	36%
HDM	20	8	6	4	2	22%	61%
Cat	13	5	4	3	1	14%	39%
Dog	2	1	1	0	0	2%	6%
Alternaria Alternata	1	0	1	0	0	1%	3%
Aspergillus Funigatus	0	0	0	0	0	0%	0%

HDM, house dust mite

Chapter 5

Exercise-induced bronchoconstriction in recreational adolescent athletes

Manuscript in preparation

Janne Goossens, Josefien Vandekerckhove, Anne-Charlotte Jonckheere, Ellen Dilissen, Nora Marain, Toon Ieven, Barbara De Wilde, Jasmine Leus, Sophie Verelst, Marc Raes, Lieven Dupont, Dominique Bullens

1. Introduction

Regular physical exercise is strongly recommended by evidence-based guidelines as one of the most convincing means to prevent chronic diseases and maintain good health (198). Even in patients with respiratory diseases such as asthma, beneficial effects of training and rehabilitation programs are demonstrated (199). Nonetheless, intense physical training may trigger airway symptoms by high demands on the respiratory system and increasing exposure to inhalant allergen, air pollutants, irritants and adverse environmental conditions (200). Therefore, it is not surprising that athletes have an increased prevalence of various respiratory complaints, such as asthma and rhinitis (116, 201). The transient airway narrowing that occurs as a result of exercise is defined as exercise-induced bronchoconstriction or EIB (110). Intense and repeated exercise, seen in athletes, is associated with a higher risk of EIB (115). Specifically, EIB has been reported to occur frequent in swimming, endurance and winter sports.

An early diagnosis of EIB in an athlete may prevent the impaired performance by taking preventive measures (128). However, the clinical diagnosis of EIB is challenging due to the poor predictive value of self-reported respiratory symptoms and broad differential diagnosis (202). Therefore, a diagnosis of EIB needs to be objectively assessed by a provocation test. Typically, this is defined as a reduction in lung function (i.e. ≥10% fall in FEV_1(L)) post challenge (exercise or EVH) compared with pre-challenge (100). Several risk factors have been associated with the development of EIB such as atopic state and training environment (154, 203). The IOC consensus statement suggested that there may be a need to screen asymptomatic athletes as part of periodic health assessments of athletes, and a diagnosis is required for athletes that may present with symptoms suggestive of EIB (204). Up to now, there is no validated screening test available to predict a positive provocation test (205). Different adjunct test may be considered during clinical approach to detect subjects at risk and evaluate EIB in athletes including AQUA© questionnaire, testing for allergic sensitisation, FeNO, respiratory symptoms during/after exercise or markers of epithelial damage (202, 205). The aim of this study was to investigate respiratory symptoms and EIB in intense adolescent recreational athletes, who perform at least 12 hours of sport a week. In addition, we wanted to identify baseline factors associated with a positive EVH test in adolescent athletes such as symptoms during exercise, atopy, markers of inflammation and epithelial damage.

2. Material and Methods

Subjects

Recreational athletes, between 12 and 18 years, who perform at least 12 hours of sports a week were recruited amongst different youth sport branches in Belgium. This cut-off of 12 hours was based on our previous observation that the median training hours a week was 12 hours in young elite athletes (12-13 years) at 'Elite Sports Schools' (101). They participated in Leuven (UZ Leuven), Hasselt (Jessa Hospitals) or Ghent (AZ Maria Middelares) between October 2019 and December 2022. Written informed consent was obtained from all participants.

Study design

The study protocol was approved by the Ethical Committee of KU/UZ Leuven after consul taken of all local Ethical Committees (S61602) and is registered on clinicaltrials.gov (NCT04103632). This study consists out of one visit. During this visit, athletes performed FeNO measurement, spirometry, followed by the eucapnic voluntary hyperventilation (EVH) test. Salbutamol (400 µg) was given after the last spirometry post EVH and a skin prick tests (SPT) were performed. In addition, a blood sample was collected and different questionnaires were filled in (see below). Participants on inhaled corticosteroids, long-acting beta2-agonists (LABA), leukotriene antagonists and antihistamines were asked to stop their medication 48 hours before the test. Short-acting beta2-agonists (SABA) were allowed until 8 hours for the study visit. Furthermore, participants were asked to not perform any sport activities on the day of the study visit.

FeNO

FeNO levels were detected with the electrochemically based NO-measuring device "Niox Vero" (NIOX Group PLC, Circassia AB, UK) and recorded in parts per billion (ppb) according to ATS/ERS guidelines (206).

Spirometry & EVH test

Spirometry was performed to determine baseline lung volumes according to ERS/ATS guidelines (207) and to calculate target ventilation during EVH test. Predicted values were calculated based on the GLI reference values (208). The EVH test was carried out in accordance with ATS recommendations and modified for this young age group (EucapSys, SMTEC, Switzerland) (101, 130). Athletes inhaled a dry air mixture containing 5% CO_2 for 6 minutes at

room temperature. A target ventilation of 21 x FEV_1, representing 70% of maximal voluntary ventilation (MVV), was required to be maintained (130). If the FEV_1 drops 10% or more at one of the time points (1', 5', 10', or 15') following the EVH test, the test was considered to be positive (the measurement after 1' was ignored). Classification into mild (10 – 25% fall in FEV_1) and moderate (25 – 50% fall in FEV_1) EIB was applied (202). In addition, the AUC for fall in FEV_1 over measured timepoints post EVH test was determined). Reversibility testing was carried out using 400 µg of salbutamol following the last spirometry post EVH test and compared with this last spirometry (15' post EVH test). Lung function was assessed 15' later.

SPT

SPT for 10 common aero-allergens was performed on all subjects: grass pollen, mugwort, plantain, birch pollen, hazel pollen, house dust mite (*Dermatophagoides pteronyssinus*), cat, dog, *Alternaria Alternata*, *Aspergillus fumigatus* (HAL Allergy, Leiden, The Netherlands). An athlete was considered to be atopic if at least one allergen was positive (≥ 3 mm and larger than the negative control) (209).

Questionnaires

The AQUA© questionnaire (155, 156) as well as the asthma control test (ACT) (210) and asthma control questionnaire (ACQ) (211) were filled in by all subjects. Additionally, a self-made exposure questionnaire based on previous cohort studies (99, 101) was filled in to assess presence of airway symptoms (e.g. wheezing, dyspnoea, coughing, rhinorrhoea, …), medication use, family history of allergies, exposures (pets, smoking, …) and hours of sports a week. Based on the reported sport discipline and hours of sport a week inside and outside, athletes were subdivided into 'indoor' if most of the sport activities were inside, 'outdoor' if most of the sport activities were outside and 'swimming' if the athlete was member of official Flemish swimming club and participating at swimming competitions. An athlete was considered to be asthmatic if the athlete confirmed that asthma was diagnosed by physician with exclusion of pre-school wheezing (≤ 6 years).

Blood samples

Club cell protein (CC16) (Biovendor, Brno, Czech Republic), undiluted; surfactant protein D (SP-D) (R&D Systems, Minneapolis, United States), 1/5 diluted; and high mobility group box 1 (HMGB-1) (Abbexa, Cambridge, United Kingdom), 1/20 diluted; were measured in serum samples using immunoassays according to manufacturer's guidelines.

Statistics

A power calculation was performed based on previous study in first grade elite athletes (101), in which atopy was present in 35% of the subjects and EVH test was positive in 27% of these atopic subjects and 12.5% in non-atopic subjects. Therefore, a sample size of 330 athletes (type I error: 0.05; type II error: 0.10; power: 90%) should be sufficient to meet this objective. Statistical analysis was performed with Graphpad Prism v.9 (San Diego, California, USA). Normality was assessed with Shapiro-Wilk test. A Chi-square test or Fisher exact test was used to evaluate proportions and correlations were evaluated with a Spearman or Pearson correlation test, depending on the distribution of the data. To compare two groups, unpaired t-test was used for parametric data and Mann-Whitney test for non-parametric data. To compare more than 2 groups, non-parametric Kruskal-Wallis with Dunn's multiple comparisons test or parametric One-Way ANOVA with Bonferroni's multiple comparisons test was used. Multiple regression analysis was performed with SPSS v.28. If p was < 0.05 data were considered significantly different.

3. Results

Subject characteristics

In total, 327 athletes between the age of 12 and 18 years old (median age: 15.8), performing at least 12 hours of sport a week, participated in the study and met the inclusion criteria (figure 1). Participant characteristics are summarized in table 1. Athletes were included amongst different sport disciplines: winter sports (n = 3), rowing (n = 4), weight lifting (n = 6), martial arts (n = 9), triathlon (n = 11), dance (n = 11), hockey (n = 14), multi sports (n = 20), track and field (n = 27), soccer (n = 34), gymnastics (n = 36), (indoor) field sports (n = 43), cycling (n = 53) and aquatic sports (n = 56) (figure 2).The percentage of male participants was 57%. The median training hours a week was 14 hours and athletes had a median intense training history (with ≥ 10 hours of sport/week) for 4.5 years. One hundred and thirty athletes (40%) were sensitized to at least one allergen on SPT of which 79 athletes had multiple sensitisations (61%). The most common aeroallergen was house dust mite (n = 77), followed by grass pollen (n = 71) and birch (n = 57).

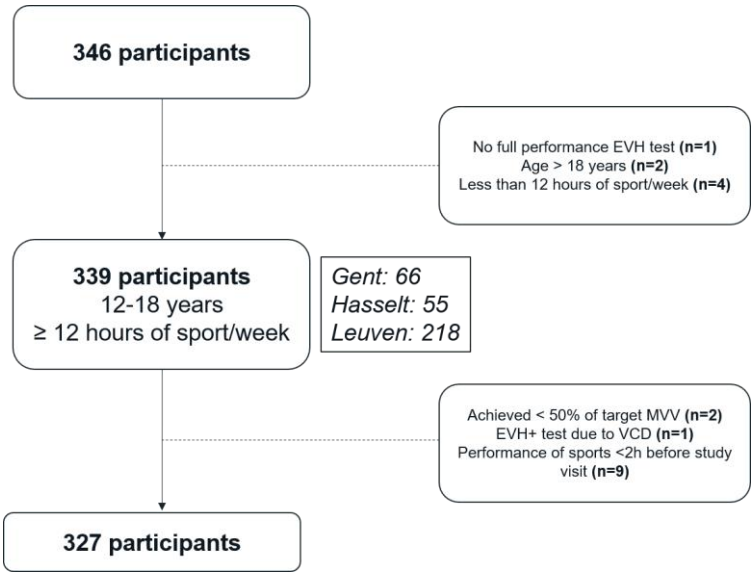

Figure 1: Included participants. Recreational athletes were recruited amongst 3 centres: Gent (AZ Maria Middelares), Hasselt (Jessa Hospitals) and Leuven (UZ Leuven). Participants needed to be between the age of 12 and 18 years old and perform at least 12 hours of sports a week. To ensure reliability of the results, athletes with less than 50% of target MVV were excluded. In addition, vocal cord dysfunction (VCD) was diagnosed by physician in an athlete with positive EVH test and was therefore excluded. Finally, although asked to not perform sport activities on the day of the study visit, some athletes did perform sport activities within 2 hours before study visit and were excluded from the analysis due to refractory period which may influence the outcome of EVH test.

Table 1: Subject characteristics. Data are represented median with interquartile range.

Number (n)	327	
Age (years)	15.8 (14.2 – 16.9)	
Gender (m/f)	185/142	
Height (cm)	m: 174 (164 – 180)	f: 164 (160 – 168)
Mass (kg)	m: 60.0 (50.1 – 69.7)	f: 55.7 (50.0 -62.0)
BMI (kg/m^2)	m: 19.7 (17.8 – 21.6)	f: 20.3 (18.7 – 22.0)
Atopic Of which multiple sensitisations	130 (40%) 79 (61%)	
Allergy diagnosis	106 (32%)	
EIB n(%)	72 (22%)	
Asthma diagnosis	45 (14%)	
FEV$_1$ (L)	3.6 (3 – 4.2)	
FEV$_1$% predicted	101 (92 – 109)	
FVC (L)	4.17 (3.6 – 4.9)	
FVC% predicted	111 (103-140)	
TI (%)	85 (81 – 90)	
FeNO (ppb)	15 (10 – 26)	
Hours of sport/week	14 (12 – 17)	
Training years	4.5 (2.8 – 6.2)	
Type of training Indoor n(%) Outdoor n(%) Swimming n(%)	 115 (35%) 156 (48%) 56 (17%)	

EIB, exercise-induced bronchoconstriction; TI, Tiffeneau index (FEV_1/FVC)

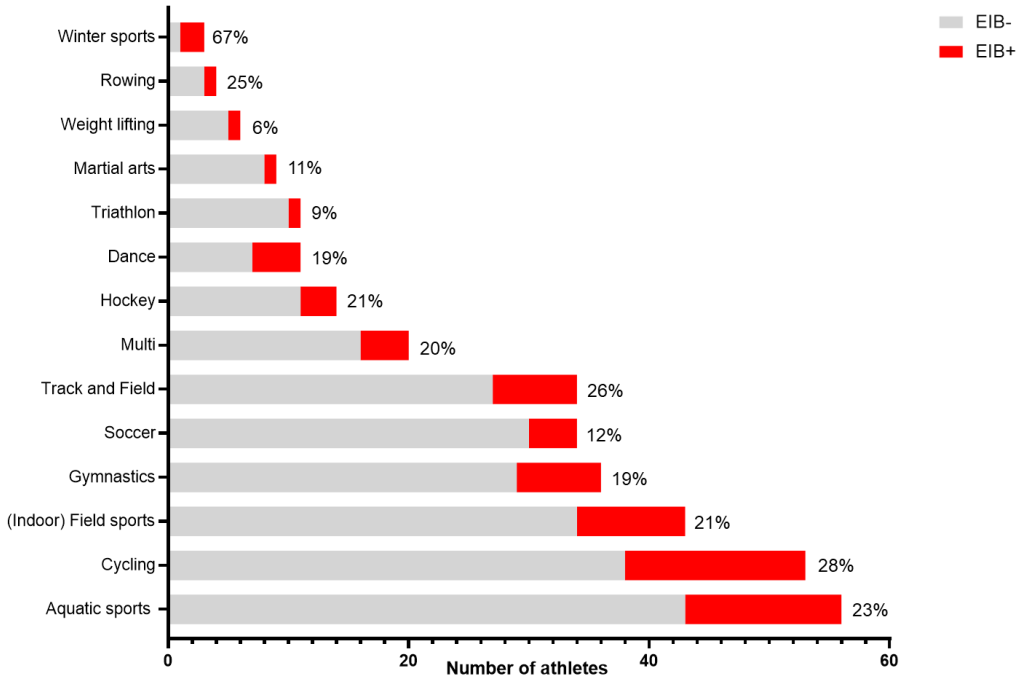

Figure 2: Overview of included sport disciplines. Percentage of EIB in each sport discipline is indicated. For overview of included sport disciplines and percentage EIB without asthma see figure E1.

Upper and lower respiratory symptoms

The included athletes were divided into subgroups based on the type of training: indoor (n = 115), outdoor (n = 156) and swimming (n = 56). Lower airway symptoms were reported in athletes during sport activities (figure 3A) with more than 30% reporting shortness of breath during exercise. Wheezing and coughing during exercise were most common in swimmers (32% and 35% respectively). Upper respiratory symptoms were often present in athletes without specific association to sport activities (figure 3B). Particularly rhinorrhoea was most common during exercise ranging from to 12 to 18 % of athletes amongst the different sport disciplines.

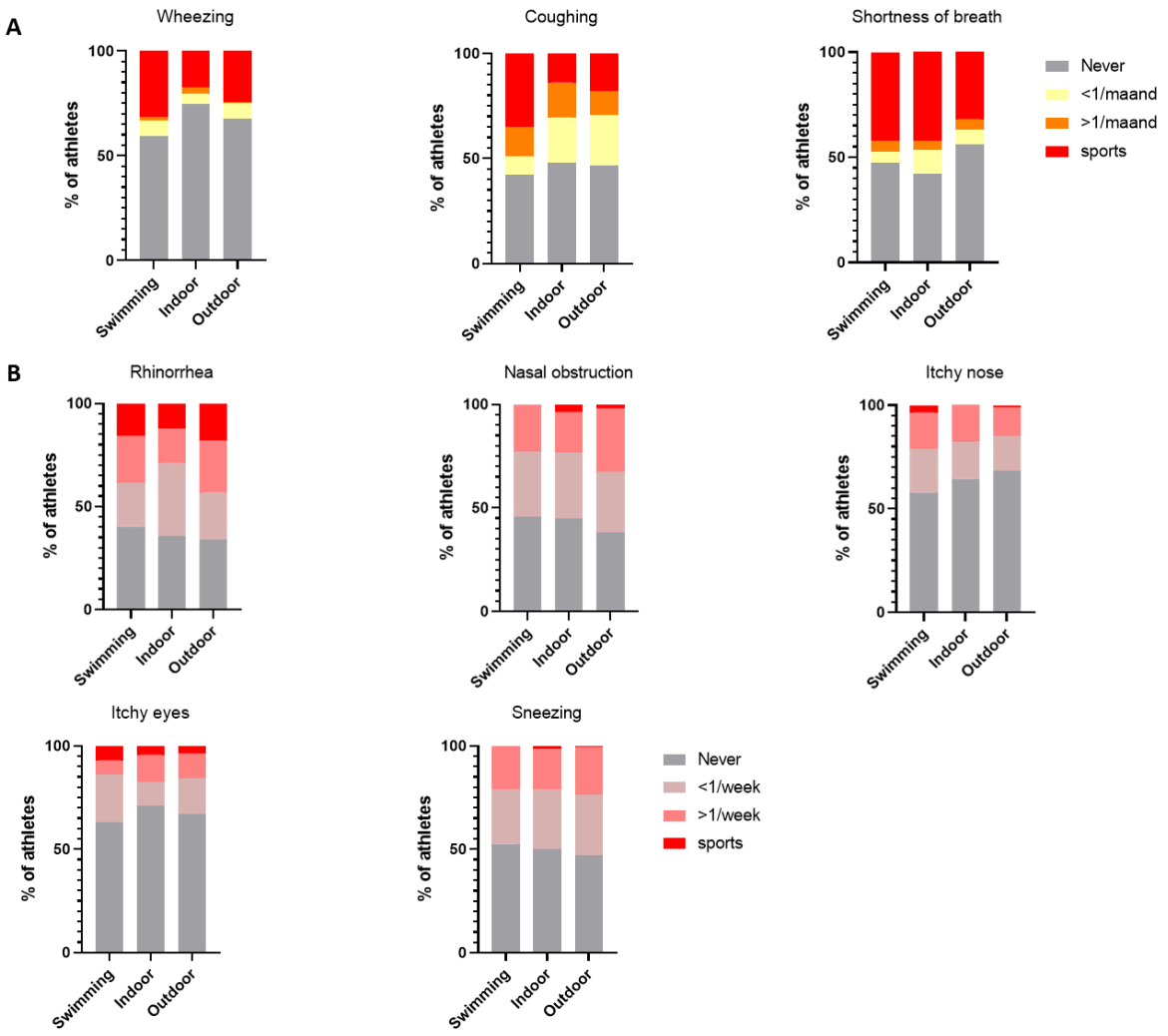

Figure 3: Reported airway symptoms. (A) Upper respiratory symptoms reported in athletes including wheezing, coughing and shortness of breath: < 1/month, >1x/month or during sport activities. **(B)** Lower respiratory symptoms reported in athletes including rhinorrhea, nasal obstruction, itchy nose, itchy eyes and sneezing: <1/week, >1/week, during sport activities.

Lung function

Focusing on the lung function amongst the defined sport disciplines, FVC% and FEV_1% were significantly elevated in swimmers compared to indoor and outdoor athletes, respectively (figure 4A, B), while the Tiffeneau index was not significantly different amongst the sport disciplines (figure 4C). In contrast, FeNO levels were significantly elevated in outdoor athletes compared to indoor athletes (figure 4D). The same results were obtained after excluding athletes with asthma (figure E2).

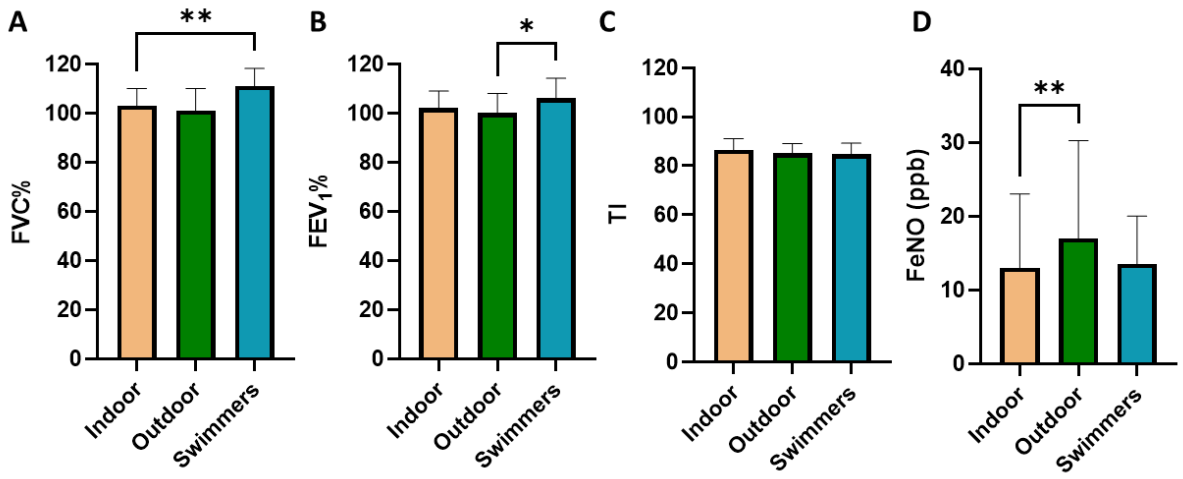

Figure 4: Lung function parameters. FVC% **(A)**, FEV_1% **(B)**, Tiffeneau index (TI) **(C)**, and FeNO levels **(D)** compared between indoor athletes (n=115), outdoor athletes (n=156) and swimmers (n=56). Kruskal-Wallis test with Dunn's multiple comparisons test. *p<0.05, *p<0.01.

EIB and asthma diagnosis

EIB diagnosis was found in 72 athletes (22%). Concretely, 23% of swimmers, 22% of outdoor and 21% of indoor athletes received a positive EIB diagnosis (figure 5A). Besides, 45 adolescent athletes (14%) mentioned a physician diagnosed asthma diagnosis (pre-school wheezing excluded), including, 9% of swimmers, 19% of outdoor en 10% of indoor athletes (figure 5A). Concretely, 55 EIB^+ athletes (76%) did not have a prior diagnosis. For EIB proportions excluding prior asthma see figure E3. The highest proportions of EIB were found in winter athletes and cyclists, while the lowest proportions were found in weight lifting and martial arts (figure 2). However, no significant association with EIB diagnosis was observed for indoor versus outdoor, aquatic versus non-aquatic athletes or endurance versus non-endurance athletes (see supplementary figure E4). A mild EIB response (10 – 25% fall in FEV_1) was found in 55 athletes, of which 11 athletes (20%) mentioned a prior asthma diagnosis. While a moderate EIB response (25 – 50% fall in FEV_1) was found in 17 athletes, of which 6 (35%) athletes mentioned a prior asthma diagnosis. Looking at the use of inhalation medication (including SABA/LABA and/or ICS), we found 58 athletes with a positive EVH test without any inhalation medication (figure 5B). Within the group of athletes who received inhalation treatment, 17 athletes had a negative EIB test. Finally, inhalation medication was used in a small group of athletes (n = 6) without asthma and/or EIB diagnosis. Specifically, this group includes endurance sports: cyclists (n = 3), swimmers (n = 2) and rowing (n = 1). Overview of specific inhalant or nasal corticosteroid use can be found in figure E5.

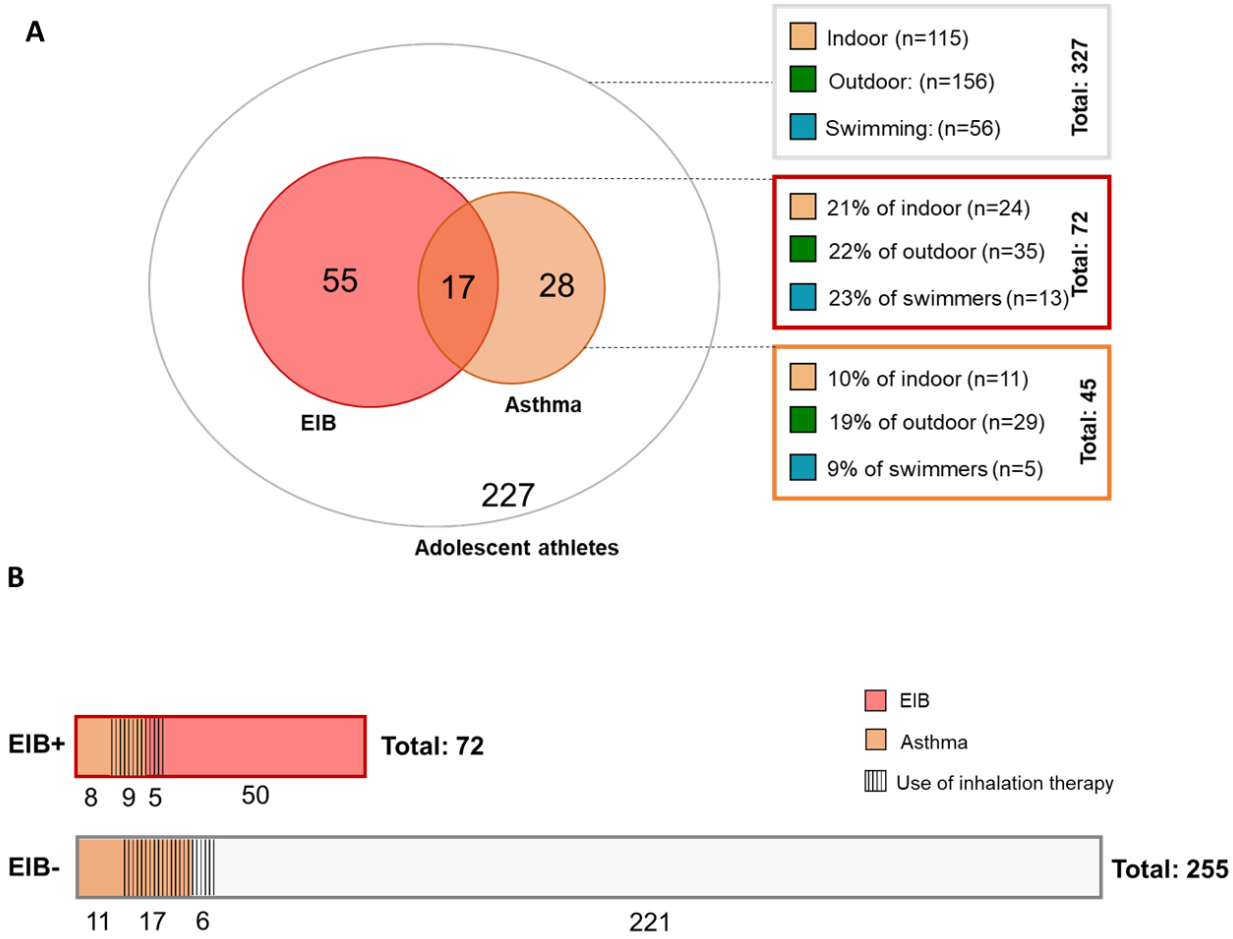

Figure 5: EIB and asthma diagnosis in adolescent athletes. (A) Distribution of athletes with EIB diagnosis (n=72) and asthma diagnosis (n=45) amongst recreational adolescent athletes. **(B)** EVH test outcome and treatment in athletes with and without asthma.

Asthma diagnosis

We observed a significant association of having an asthma diagnosis and a household member who smokes (p=0.0323). Specifically looking at the group of athletes with an asthma diagnosis (n = 45), athletes with suspected typical Type 2 inflammation (defined as athletes with previous allergy diagnosis or at least one positive SPT result)(n = 34, 75%) and suspected non-Type 2 inflammation (n = 11, 25%) were studied (table 2) (114, 192, 212). Athletes with suspected Type 2 inflammation had significantly higher FeNO levels and AQUA© score compared to suspected non-Type 2 asthma (p=0.0025 and p=0.0004). We did not find a significant association between suspected non-Type 2 asthma and the type of sport or training intensity (training years and hours of sport a week). On the other hand, we found a significant association of asthma diagnosis and performing outdoor sport discipline (19% of outdoor athletes vs 9% of non-outdoor athletes, p=0.0165).

Table 2: Characteristic of adolescent athletes with suspected Type-2 and non-Type 2 asthma (114, 192, 212)**.**

	Type 2 asthma	Non-Type 2 asthma	P-value
Number (n)	34	11	
EIB+ n (%)	7 (21%)	2 (18%)	0.9999
Endurance n (%)	17 (50%)	3 (27%)	0.2972
Type Indoor Outdoor Swimmers	 7 (21%) 23 (67%) 4 (12%)	 4 (36%) 6 (55%) 1 (9%)	 0.6420
Training hours/week	13 (12 - 16)	14 (12 - 18)	0.5922
Training years	5.1 (3.5 – 6.3)	6.6 (3.4 - 10.9)	0.3637
Wheezing during exercise▲	20 (59%)	6 (54%)	0.9999
Shortness of breath during exercise▼	25 (74%)	10 (91%)	0.4087
FeNO (ppb)	26 (16 – 46)	12 (8 – 15)	**0.0025**
AQUA score	18 (13 – 21)	7 (4 – 14)	**0.0004**
ACT score	20 (18 - 25)	23 (22 - 24)	0.2253
ACQ score	0.5 (0 – 1.3)	0.5 (0 – 0.7)	0.5801
Household member who smokes	8 (24%)	1 (9%)	0.4157

Data is represented as mean with standard deviation for parametric data and median with interquartile range for non-parametric data and analysed with unpaired t-test or Mann-Whitney test, respectively. Categorical variables were analysed with Chi-square test.

▲Wheezing during exercise was assessed with following question: 'Do you suffer from wheezing during exercise?'

▼Shortness of breath was assessed with following question: 'Do you feel short of breath during exercise?'

Validation atopy – FeNO – AQUA

This independent cohort of adolescent athletes was used to validate our previous obtained results on the ability of AQUA© and FeNO to predict atopy (table E1). Indeed, FeNO and AQUA© were able to predict atopy with AUC of 0.764 ($p<0.0001$) and 0.734 ($p<0.0001$), respectively. A cut-off of 15 ppb had a sensitivity of 75% and specificity of 61% ($p<0.0001$). For an AQUA© score of 6, we found a sensitivity of 84% and specificity of 46% ($p<0.0001$). The sensitivity of the cut-off of 15 ppb increases to 80% to detect multiple sensitisations, with a specificity of 54% ($p<0.0001$). Also within in the group of asthmatic athletes we found an excellent sensitivity of 92% and a specificity of 63% ($p=0.0002$) to predict atopy.

Associations with EIB

We next assessed whether atopy and other factors are associated to EIB in our cohort (table 3). The age of the EIB^+ athletes was significantly lower compared to EIB^- athletes. We furthermore found significantly lower Tiffeneau index and more athletes with a previous asthma diagnosis in the group of athletes with EIB. The reversibility post EVH test was significantly higher in athletes with EIB compared to athletes without EIB. Furthermore, this

reversibility significantly correlated with maximal fall in FEV_1(%) within EIB^+ athletes (r=-0.4416, p=0.0001) and with AUC post EVH test (r=0.5111, p<0.0001). Considering symptoms during exercise, both shortness of breath and wheezing were associated with EIB. In addition, being atopic, mono- or with multiple sensitisations or having a previous allergy diagnosis was significantly associated with EIB. Also, our previously defined predictors of atopy, AQUA© and FeNO were significantly associated with EIB. Finally, ACQ score was significantly lower in EIB^+ athletes compared with EIB^- athletes.

Table 3: Characteristics of adolescent athletes with EIB and without EIB.

	EIB-	EIB+	P-value
Number	255	72	
Gender (m/f)	138/117	47/25	0.1065
Age (years)	15.6 ± 1.8	15.1 ± 1.8	**0.0455**
BMI (kg/m^2)	20.6 ± 3.0	20.0 ± 2.7	0.0929
Lung function			
FVC% predicted	102.2 ± 12.8	104.4 ± 13.3	0.3713
FEV_1% predicted	100.7 ± 13.3	100.3 ± 13.4	0.5156
TI (%)	85.7 ± 6.6	83.2 ± 6.2	**0.0048**
Reversibility post EVH test (%)	4.3 (1.2 – 7.2)	12.0 (5.8 – 18.4)	**<0.0001**
Achieved target ventilation (%)	103.1 ± 20.6	102.1 ± 17.0	0.6991
AUC post EVH test	43 (17 - 68)	184 (129 – 244)	**<0.0001**
Asthma diagnosis	31 (12%)	17 (24%)	**0.0226**
Symptoms during exercise			
Shortness of breath▼	103 (40%)	40 (56%)	**0.0309**
Wheezing▲	78 (31%)	28 (39%)	**0.0308**
Atopic state			
Atopic	160 (63%)	56 (78%)	**0.0171**
Multiple sensitisations	49 (19%)	30 (42%)	**0.0002**
Allergy diagnosis	76 (30%)	34 (47%)	**0.0073**
AQUA score	8 (3-14)	12 (6-17)	**0.0010**
FeNO (ppb)	14 (10-23)	22 (12-45)	**0.0006**
Frequent upper airway infection	56 (22%)	15 (21%)	0.9999
Allergic family member	173 (68%)	50 (69%)	0.9999
Serum markers epithelial damage			
CC16 (pg/mL)	7.40 (5.58-9.83)	6.53 (4.90-8.34)	0.0768
HMGB1 (pg/mL)	2520 (1528-4560)	2984 (1478-5686)	0.4330
SP-D (ng/mL)	6.52 (4.86-8.14)	7.21 (5.66-9.55)	0.0631
ACQ	25 (24-25)	24.5 (22-25)	**0.0041**
ACT	0 (0-1)	0 (0-2)	0.0582

Data are shown as mean with standard deviation (normal distributed) or median with interquartile range (not normal distributed) and analysed with unpaired t-test or Mann-Whitney test, respectively. Categorical variables were analysed with Chi-square test. EIB, exercise-induced bronchoconstriction; TI, Tiffeneau index

▲Wheezing during exercise was assessed with following question: 'Do you suffer from wheezing during exercise?'

▼Shortness of breath was assessed with following question: 'Do you feel short of breath during exercise?'

Performance of identified associations

Significant associations were identified between EIB diagnosis and previous asthma diagnosis, symptoms during exercise (shortness of breath and wheezing), atopic state, allergy diagnosis, AQUA© score, FeNO levels and ACQ score. Next, we wanted to assess the individual effectiveness of each factor to predict EIB (table 4). For the analysis without asthmatics (n = 48), see table E2. The highest sensitivity was found for AQUA© scores ≥ 5 and ≥ 6 (81%, p=0.0207 and 78%, p=0.0171). Whereas the highest specificity was found for wheezing during exercise (82%, p=0.0002), followed by multiple sensitisations (81%, p=0.0002) and FeNO ≥ 25 ppb (78%, p=0.0003). Furthermore, FeNO negatively and significantly correlated with maximal fall in FEV_1 post EVH test (r=-0.2735, p=0.0056) and with AUC post EVH test r=0.3476, p=0.0028) in atopic athletes specifically, indicating that athletes with higher FeNO levels have a deeper fall in FEV_1. Combing these factors in a multiple logistic regression analysis with EIB diagnosis as independent variable, we found wheezing during exercise and FeNO to be significant factors within athletes without asthma (n = 282) and the whole group of athletes (n = 327), respectively (table E3, E4). In both regression analyses, age and BMI were considered to be significant factors in the outcome of EVH test.

Table 4: Predictive factors for a diagnosis of EIB in athletes.

	Odds ratio	95% CI	Sensitivity	Specificity	P-value
Symptoms during exercise					
Shortness of breath▼	1.8	1.1 – 3.1	56%	60%	**0.0309**
Wheezing▲	3.0	1.7 – 5.2	40%	82%	**0.0002**
Atopic state					
Mono	2.1	1.2 – 3.6	54%	64%	**0.0062**
Multiple	3.0	1.7 – 5.3	42%	81%	**0.0002**
Allergy diagnosis	2.1	1.2 – 3.6	46%	71%	**0.0069**
AQUA score					
≥ 5	2.1	1.1 – 3.9	81%	34%	**0.0207**
≥ 6	2.1	1.1 – 3.8	78%	37%	**0.0171**
FeNO					
≥ 15 ppb	2.2	1.2 – 3.8	68%	51%	**0.0051**
≥ 20 ppb	2.4	1.4 – 4.2	54%	67%	**0.0014**
≥ 25 ppb	2.8	1.6 – 4.9	44%	78%	**0.0003**
Asthma diagnosis	0.4	0.2 – 0.9	12%	76%	**0.0226**

P-value, odds ratio, 95% CI, sensitivity and specificity were determined via a Fisher's exact two-tailed test.
▲Wheezing during exercise was assessed with following question: 'Do you suffer from wheezing during exercise?'
▼Shortness of breath was assessed with following question: 'Do you feel short of breath during exercise?'

Serum epithelial damage markers

Markers of epithelial damage were measured in collected serum samples of EIB^+ athletes (n = 67) and similar number of EIB^- athletes (n = 77). There was no significant difference in serum CC16, HMGB1 and SP-D levels in EIB^+ athletes compared with EIB^- athletes (table 3). Moreover, significantly different serum levels of CC16 were measured amongst different sport disciplines, with highest levels measured in swimmers (median: 8.2 pg/mL, p=0.0210)(figure 6A). Furthermore, serum HMGB1, a DAMP released after epithelial damage, significantly and positively correlated with the number of intense training years (r=0.2654, p=0.0013)(figure 6B), driven by outside athletes (table E5). Besides, serum SP-D levels were significantly different amongst EIB^- athletes, athletes with moderate EIB and athletes with mild EIB (7.4 pg/mL vs 4.5 pg/mL, p=0.0429)(figure 6C).

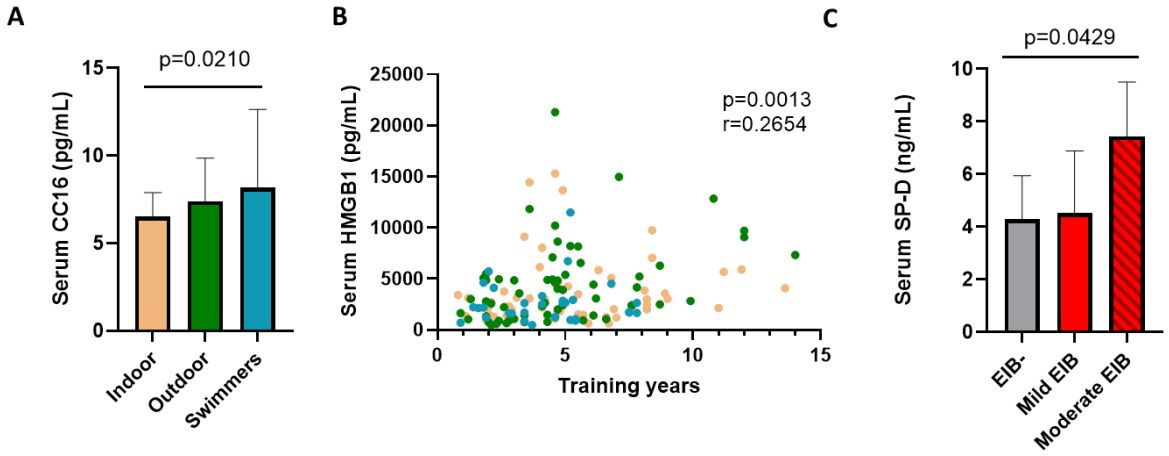

Figure 6: Serum epithelial damage markers in adolescent athletes. (A) CC16 levels (pg/mL) in indoor, outdoor athletes and swimmers. **(B)** Correlation between training years and serum HMGB1 (pg/mL) in outdoor (green), indoor (yellow) athletes and swimmers (blue). **(C)** Serum surfactant D (SP-D) (ng/mL) levels in EIB- athletes, mild (10 - 25% max. fall in FEV_1) EIB+ athletes and moderate (25 – 50% max. fall in FEV_1) EIB+ athletes. Kruskal-Wallis test, Spearman correlation, Kruskal-Wallis test. CC16, club cell protein; HMGB1, high mobility group box 1; SP-D, surfactant protein D.

4. Discussion

In this study, 14% of adolescent athletes reported an asthma diagnosis, 40% of athletes were atopic and 22% of athletes tested positive for EIB. Of these EIB^+ athletes, 80% of athletes were not treated with any inhalation therapy and 76% did not receive a prior asthma diagnosis, which is often used interchangeably in real-life practice to describe a similar clinical entity of EIB. These data indicate that there might be a need to better identify athletes at risk for EIB. Investigating different factors linked to EIB, the highest sensitivity was found for an AQUA© score of 5 and 6 and highest specificity was found for reporting wheezing during exercise, multiple sensitisations and FeNO ≥ 25 ppb. Serum markers of epithelial damage were not able to differentiate EIB^+ and EIB^- athletes, but were associated to training type, training intensity and EIB severity.

Elite athletes frequently report respiratory symptoms (201). Also, this population of recreational adolescent athletes reported specific symptoms of lower airways during exercise, including shortness of breath, coughing and wheezing. The highest symptom rate was found for swimmers. These symptoms can be related to the external environment surrounding the athletes like exposure to chorine by-products for swimmers (213).

Asthma was found in 14% of included adolescent athletes and was significantly associated with outdoor athletes. In line, we found significant elevated FeNO levels in outdoor athletes compared to other sport disciplines. The prevalence of self-reported asthma among elite athletes described in literature is about 17% (214). As suggested in literature, athletes with asthma can divided into 2 different endotypes: Type 2 and non-Type 2 inflammatory disease (114, 212). Similarly as Rasmussen *et al.* we were not able to identify any significant associations between suggested non-Type 2 asthma and endurance sports (192). Suggested Type 2 asthma was the most frequent asthma endotype (75%) with elevated AQUA© score and FeNO levels compared with suggested non-Type 2 asthma. However, 25% of the athletes had non-Type 2 asthma, which is higher than expected in this young population. This suggests that intense training in young athletes may be in relation to asthma development. Furthermore, we were able to find a significant association between asthma diagnosis and having a smoking household member in our young athletes, which is in line with the fact that household smoking is considered to be a risk factor for childhood asthma in general (215).

EIB diagnosis was found in 22% of athletes, which is in line with the meta-analysis of Price *et al.* investigating lower airway dysfunction in athletes (15 -65 years)(189). Indeed, the lowest incidence was found in weight lifting and martial arts versus winter sports and cycling. Several studies already showed a high prevalence of atopy in athletes and indicated that this might be a risk factor for asthma and/or EIB (153, 216, 217). In our cohort of adolescent athletes, we indeed found a significant association between atopy and EIB. However, when athletes with prior asthma diagnosis were excluded from the analysis, this association was no longer significant. This suggests that atopy might be associated specifically to Type 2 inflammation seen in most of asthma patients. Of note, this can also be due to the relatively low number of subjects remaining in the group of athletes without asthma. Both, FeNO and AQUA© questionnaire, were validated in this study to be used as screening tool for atopy in young athletes, obtaining similar results for FeNO and even better sensitivity for AQUA©.

Surprisingly, we did not find a significant association between having EIB and the type of training. In literature it is widely accepted that high risk sports are endurance sports with elevated risk when athletes are exposed to additional external triggers like cold air or chlorine by-products (115). This could be explained by the fact that the training intensity of these recreational athletes might not yet be achieving the same effects compared to elite athletes or due to lack of power. We cannot exclude selection bias by which athletes with more respiratory symptoms could be more attracted to participate in current study protocol. Another limitation includes the lack of airway samples such as induced sputum samples to study more in depth the underlying cellular inflammation and/or mechanisms of EIB. However, although intended, due to restrictions of Covid-19 pandemic, those samples could not be collected. Finally, we are aware of the limitation that no baseline reversibility test was performed to diagnose asthma, due to practical circumstances that all tests could be performed within one study visit, enabling study accessibility for athletes to participate. Moreover, also asthma by self-reporting can be under-diagnosed in this population of young athletes.

The medical screening of young athletes does not yet include an exercise test or EVH test to diagnose EIB. It would not be cost-effective if this is performed in every athlete. Therefore, different factors were studied in association with EIB in order to refer the subject at risk for further testing. As also demonstrated by others, we found a significant association with a previous asthma diagnosis (137, 139). A significant association was found for, allergy

diagnosis, symptoms during exercise (wheezing and shortness of breath), AQUA© score, FeNO levels and multiple sensitisations. Bougault *et al.* also identified self-reported exercise-induced symptoms and sensitisation to at least 5 allergens to be predictive for EIB in soccer players (136). However, FeNO levels were not measured. We found that FeNO measurement can be helpful to identify EIB especially in atopic athletes as FeNO levels were significantly associated to EVH response in the latter specifically. Similarly, Rouhos *et al.* found only in atopic patients an association of FeNO with bronchial hyperresponsiveness measured by histamine and exercise test (218). To the best of our knowledge, this was not demonstrated before in a cohort of adolescent athletes. These results suggest that in atopic and non-atopic athletes with EIB other mechanisms are important (219). As we demonstrated in our previous study that FeNO level of ≥ 15 ppb have higher sensitivity for atopic athletes with multiple sensitisation, it is not surprisingly that FeNO levels as well as being atopic with multiple sensitisations are both significant predictors for EIB (220). Furthermore, we found in the multiple regression analysis a significant negative coefficient for age, suggesting that there might be a dropout of older adolescent athletes with EIB not achieving intended results or the EIB might be transient in nature. We found that having a higher BMI also is a risk factor for EIB in these recreational athletes.

In our previous study in elite adolescent athletes, serum CC16 levels correlated with drop in FEV_1 post EVH test (101). However, we were not able to validate this observation in this population of recreational athletes, nor in our population of early-career elite athletes (200). However, However we identified elevated levels of serum CC16 in swimmers compared with other sport disciplines. Even though the training hours a week were significantly higher in indoor athletes compared with outdoor athletes and swimmers. This suggests increased epithelial damage in swimmers, as a result of exposure to products of chlorination. Also others demonstrated increased levels of epithelial damage in competitive swimmers (99, 104). Furthermore, we identified a significant and positive correlation between serum levels of the alarmin HMGB1 and training years, indicating that longer intense training period is associated with increased alarmin release. Indeed, it was demonstrated that high-intensity exercise modulates systemic release of HMGB1 (221). Besides, serum SP-D was significantly elevated in athletes with a moderate EIB response compared with a mild EIB response. As for EIB, the underlying mechanism is thought to be mechanical stress onto the airway, this elevated marker of epithelial damage may explain a more severe response to EVH test.

In practice, specific questions regarding symptoms during exercise can easily be added to medical screening. To translate these findings, we would suggest to start with the question 'Do you suffer from wheezing during exercise?', followed by the use of AQUA© (figure 7A). As AQUA© measurement is easy and at low cost and demonstrated good sensitivity for EIB (78%), with however poor specificity (37%). Accordingly, Allen *et al.* also suggested AQUA© to be used as an initial form of assessment to rule out EIB (159). Those steps can easily be implemented to identify athletes that are more prone to have EIB. This selected group of athletes can then be referred to perform allergy testing. Atopic athletes can be referred to undergo an EVH test or exercise challenge to confirm the presence of EIB, after excluding asthma like in the algorithm of Boulet *et al.* in New England Journal of Medicine (100). Alternatively, only athletes with multiple sensitisations (figure 7B) can be referred specifically to improve specificity or FeNO levels (figure 7C) can be used to detect atopy. Alternative approaches are summarized in table E6. However, these approaches demonstrated that there is still a lack to identify non-atopic EIB^{+} athletes without wheezing. Further research is needed to better understand the underlying mechanisms in these athletes.

In conclusion, 76% of adolescent recreational athletes are still underdiagnosed for EIB and consequently not monitored for EIB. Proper identification would allow optimal management of these young athletes. To identify athletes with possible undiagnosed EIB, the question of wheezing during exercise and AQUA© questionnaire can be added to annual medical screening follow-up.

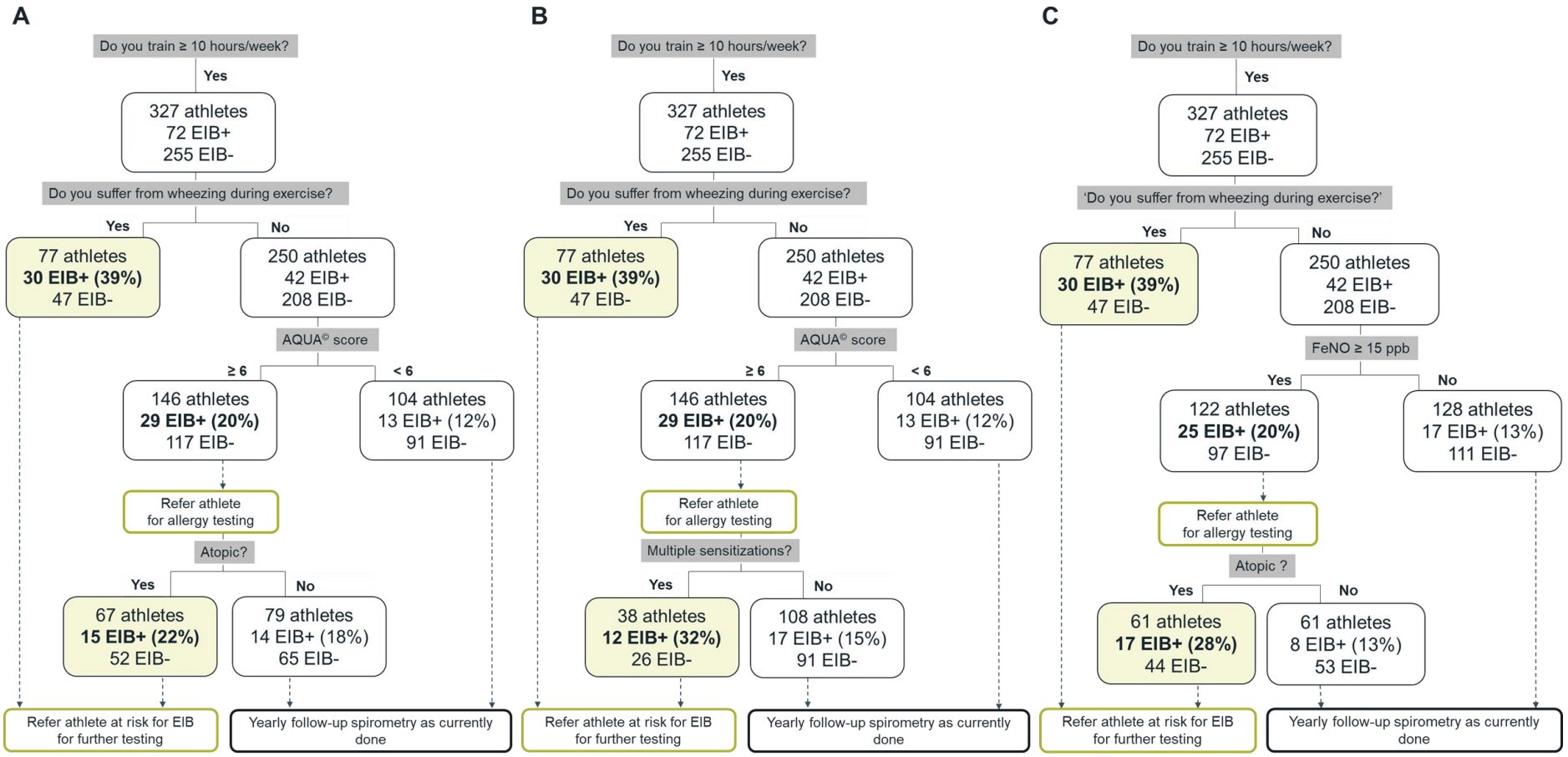

Figure 6: Suggested flow diagrams to identify EIB+ adolescent athletes. (A) 62% of EIB+ athletes were identified using 'wheeze during exercise', AQUA© questionnaire and atopy testing. Of all referred athletes for further testing, 31% will test positive. **(B)** 58% of EIB+ athletes were identified using 'wheeze during exercise', AQUA© questionnaire and multiple sensitisation testing. Of all referred athletes for further testing, 37% will test positive. **(C)** 65% of EIB+ athletes were identified using 'wheeze during exercise', FeNO and atopy testing. Of all referred athletes for further testing, 34% will test positive.

5. Supplementary material

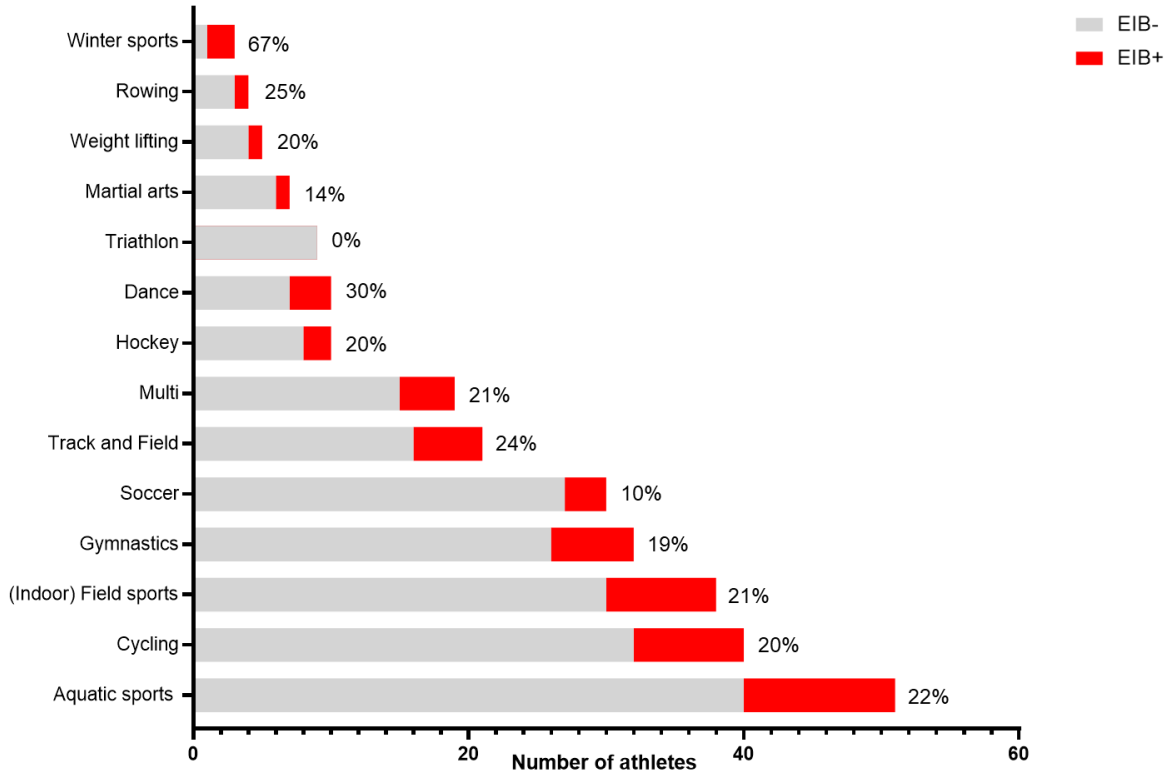

Figure E1: Overview of included sport disciplines without asthma. Percentage of EIB in each sport discipline is indicated.

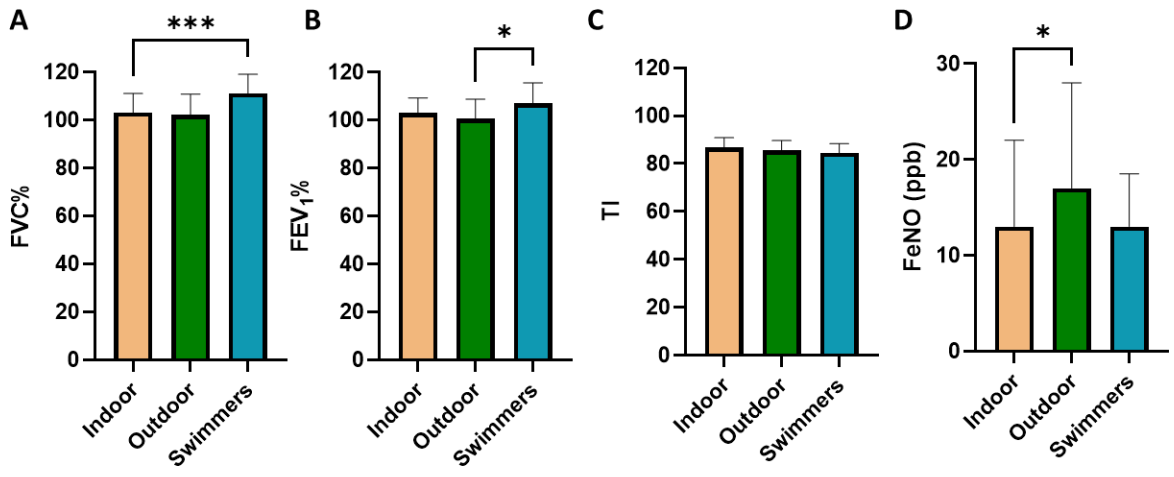

Figure E2: Lung function parameters without asthma. FVC% **(A),** FEV_1% **(B)**, Tiffeneau index (TI), **(C)** and FeNO levels **(D)** compared between indoor athletes (n=104), outdoor athletes (n=127) and swimmers (n=51). Kruskal-Wallis test with Dunn's multiple comparisons test. *p<0.05, *p<0.01.

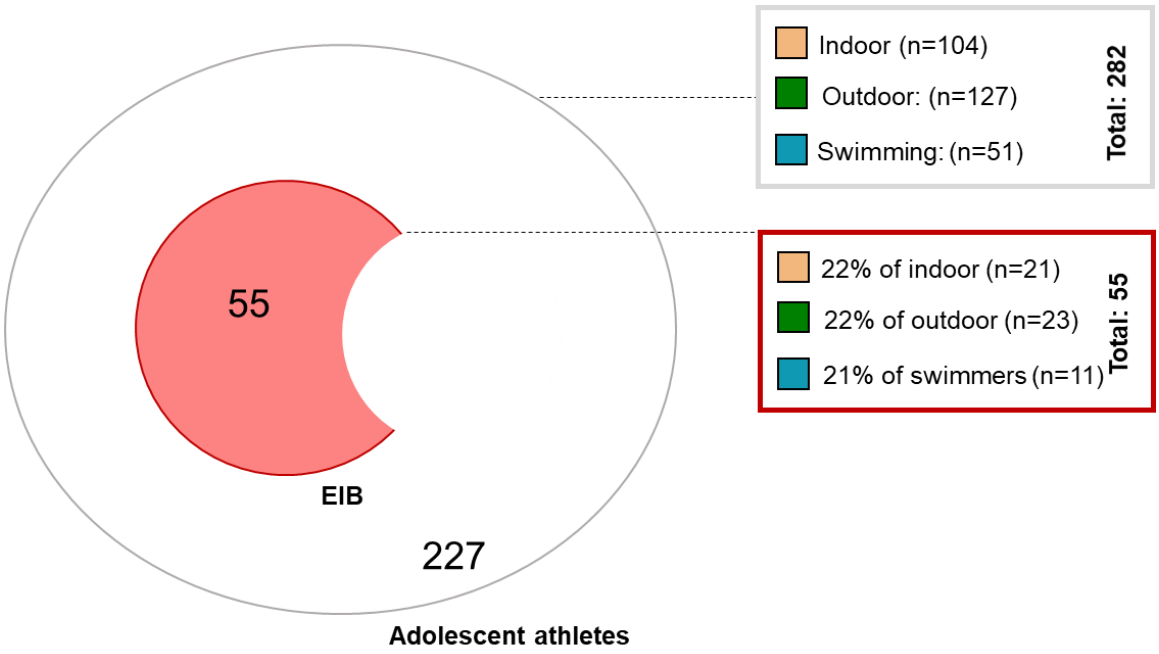

Figure E3: EIB diagnosis in adolescent athletes without asthma. Distribution of different sport disciplines amongst adolescent athletes and EIB+ athletes without asthma.

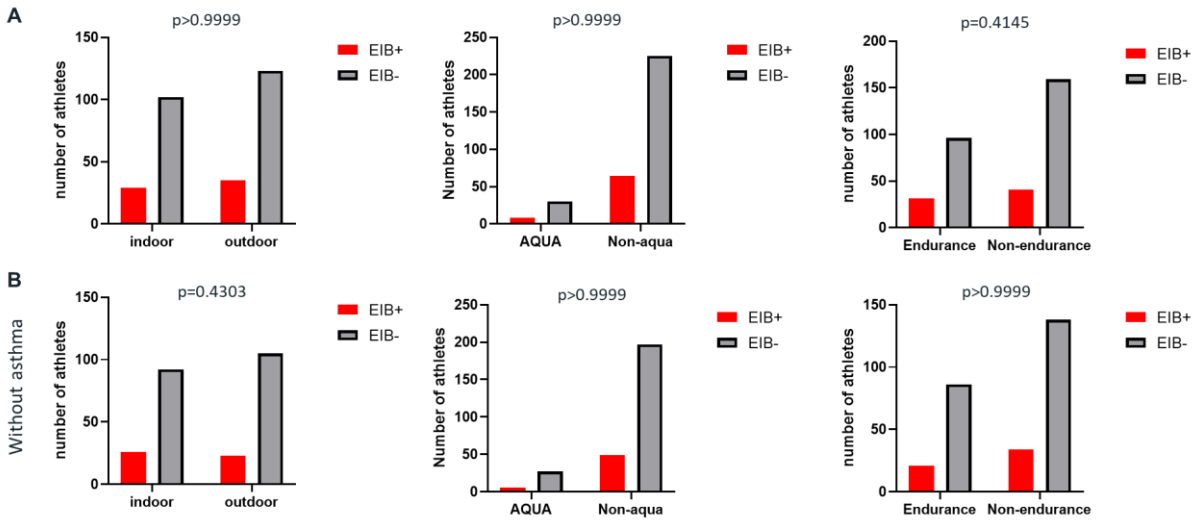

Figure E4: Associations of sport specific characteristics with EIB (A) Associations within whole group of athletes (n=327) for indoor without swimmers (n=115) versus outdoor (n=156) sport disciplines; aquatic (n=56) versus non-aquatic sport disciplines (n=271); and endurance sport (n=127) versus non-endurance sport disciplines (n=200). **(B)** Associations within athletes without asthma diagnosis (n=279) for indoor without swimmers (n=118) versus outdoor (n=128) sport disciplines; aquatic (n=33) versus non-aquatic sport disciplines (n=246); and endurance sport (n=107) versus non-endurance sport disciplines (n=172). Fisher exact test.

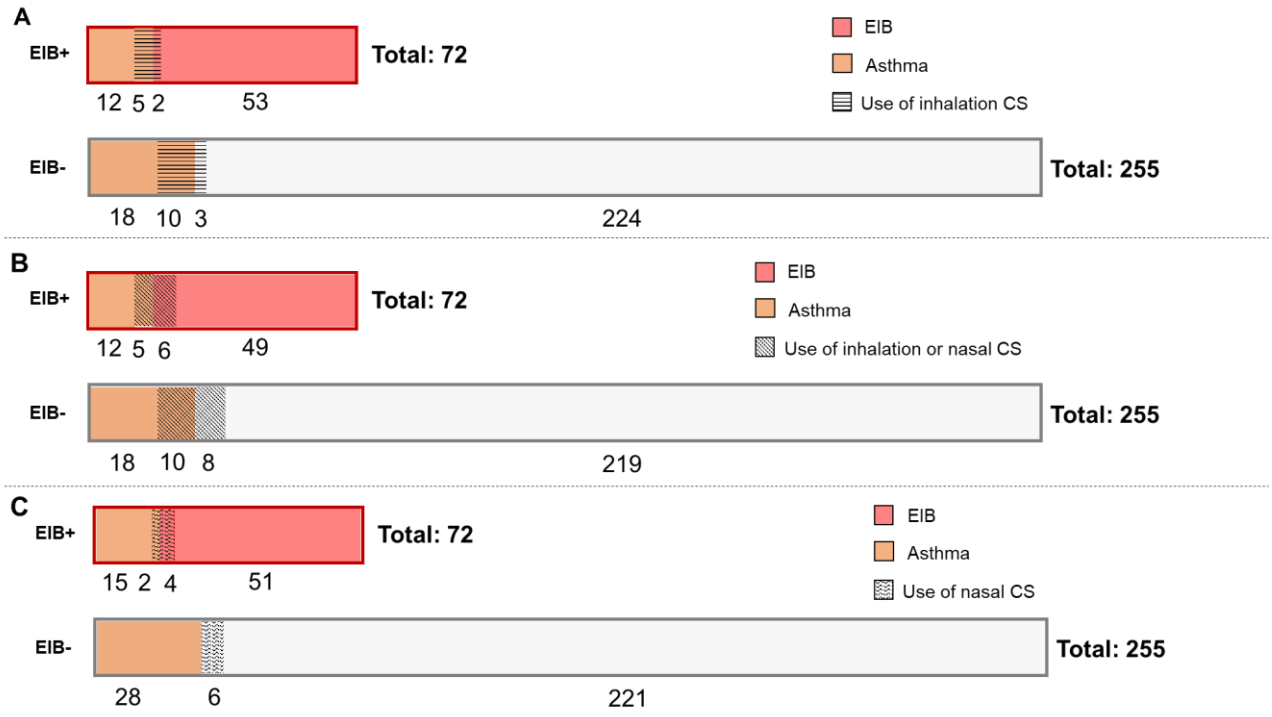

Figure E5: EVH test outcome and treatment in athletes with and without asthma. (A) Reported use of inhalation corticosteroids (ICS), **(B)** Reported use of inhalation or nasal corticosteroids (CS), **(C)** Reported use of nasal CS, expressed number of athletes.

Table E1: Diagnostic accuracy of AQUA© and FeNO for atopy

AQUA score	Atopic (n=130)	Non-atopic (n=197)	P-value	Sensitivity (%)	Specificity (%)
≥ 5	112	115	<0.0001	86	42
< 5	18	82			
≥ 6	109	107	<0.0001	84	46
< 6	21	90			
FeNO (ppb)	**Atopic (n=130)**	**Non-atopic (n=197)**	**P-value**	**Sensitivity (%)**	**Specificity (%)**
≥ 15	98	77	<0.0001	75	61
< 15	32	120			
≥ 20	81	41	<0.0001	62	79
< 20	49	156			
≥ 25	67	21	<0.0001	51	90
< 25	63	176			
FeNO (ppb)	**Atopic (≥2 sensitisations, n=79)**	**Non-atopic and atopic (1 sensitisation, n=248)**	**P-value**	**Sensitivity (%)**	**Specificity (%)**
≥ 15	63	112	<0.0001	80	54
< 15	16	136			
≥ 20	52	70	<0.0001	66	72
< 20	27	178			
≥ 25	45	43	<0.0001	59	83
< 25	34	205			

P-value, sensitivity and specificity were determined via a Fisher's exact two-tailed test.

Table E2: Predictive factors for a diagnosis of EIB in athletes without asthma.

	Odds ratio	95% CI	Sensitivity	Specificity	P-value
Symptoms during exercise					
Shortness of breath	1.9	1.1 – 3.6	51	65	**0.0308**
Wheezing	2.9	1.5 – 5.8	33	86	**0.0028**
Atopic state					
Mono	1.8	0.-9 – 3.3	47	67	0.0618
Multiple	2.6	1.4 – 5.0	36	82	**0.0054**
Allergy diagnosis	0.5	0.9 – 1.0	62	24	**0.0419**
AQUA score					
≥ 5	1.8	0.9 – 3.3	73	40	0.1190
≥ 6	1.8	0.9 – 3.3	72	40	0.1190
FeNO					
≥ 15 ppb	1.7	1.0 – 3.2	62	52	0.0734
≥ 20 ppb	2.1	1.2 – 4.0	49	69	**0.0168**
≥ 25 ppb	2.2	1.7 – 4.0	36	79	**0.0218**

P-value, odds ratio, 95% CI, sensitivity and specificity were determined via a Fisher's exact two-tailed test.

Table E3: Multiple logistic regression analysis in adolescent athletes without asthma.

	B	S.E.	Wald	df	Sig.	Exp(B)
FeNO	0.013	0.008	3.047	1	0.081	1.013
AQUA	0.009	0.027	0.106	1	0.745	1.009
Wheezing during exercise	0.852	0.383	4.944	1	**0.026**	2.344
Polysensitisation	0.065	0.128	0.258	1	0.611	1.067
Age	-0.296	0.104	8.072	1	**0.004**	0.744
BMI	0.190	0.066	8.337	1	**0.004**	1.209
Constant	-1.390	1.434	0.940	1	0.332	0.249

Independent variable: EIB diagnosis

Table E4: Multiple logistic regression analysis in adolescent athletes.

	B	S.E.	Wald	df	Sig.	Exp(B)
FeNO	0.017	0.007	6.110	1	**0.013**	1.017
AQUA	0.152	0.504	0.090	1	0.764	1.164
Wheezing during exercise	0.601	0.332	3.271	1	0.070	1.824
Polysensitisation	0.093	0.151	0.376	1	0.540	1.097
Age	-0.298	0.097	9.486	1	**0.002**	0.742
BMI	0.187	0.060	9.728	1	**0.002**	1.205
Asthma	0.618	0.407	2.297	1	0.130	1.854
Constant	-1.334	1.350	0.976	1	0.323	0.263

Independent variable: EIB diagnosis

Table E5: Correlation between serum HMGB1 and training years

Training years	Pearson r	P-value
Indoor athletes	0.2182	0.3278
Outdoor athletes	0.3953	**0.0048**
Swimmers	0.0631	0.9806

P-values were corrected by Bonferroni multiple testing.

Table E6: Alternative proposed components of flowcharts to identify EIB+ adolescent athletes.

Components flowcharts			EIB+ athletes/total referred athletes	% positive testing	% of EIB+ athletes identified
Wheezing during exercise	AQUA score ≥ 6		59/223	26	82
Wheezing during exercise	AQUA score ≥ 6	≥ 3 sensitisations	36/105	34	50
Wheezing during exercise	AQUA score ≥ 6	≥ 4 sensitisations	32/92	35	44
Wheezing during exercise	FeNO ≥ 15 ppb	Multiple sensitisations	42/111	38	58
Wheezing during exercise	Previous allergy diagnosis	FeNO ≥ 15 ppb	43/117	37	60
Wheezing during exercise	Previous allergy diagnosis	FeNO ≥ 20 ppb	40/104	38	56
Wheezing during exercise	Previous allergy diagnosis	FeNO ≥ 25 ppb	38/96	40	53

Chapter 6

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

RESEARCH ARTICLE

Manuscript published in Thorax. 2023

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

[1] Allergy and Clinical Immunology Research Group, Department of Microbiology and Immunology, KU Leuven, Belgium
[2] Sport Medical Advice Centre, UZ Leuven, Belgium
[3] Flemish Swimming Federation, Merelbeke, Belgium
[4] Sports doctor's practice, Wilrijk, Belgium
[5] Academic Centre for General Practitioners, KU Leuven, Belgium
[6] Pediatric Allergy, AZ Maria Middelares Gent, Belgium
[7] Clinical division of Paediatrics, UZ Leuven, Belgium
[8] Pediatrics, Jessa Hospital Hasselt, Belgium
[9] Laboratory of Respiratory Diseases and Thoracic Surgery (BREATHE), Department of Chronic Diseases and Metabolism, KU Leuven, Belgium
[10] Clinical division of Respiratory Medicine, UZ Leuven, Belgium

1. 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: 20-36, $p<0.0001$). Athletes had significantly higher substance P (13.3 pg/mL, 95% CI: 2.0-19.2) and calprotectin (1237 ng/mL, 95% CI: 531-2490) 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 FEV_1(%) 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 hyperreactivity.

2. Introduction

Although regular physical activity is of utmost importance to prevent worsening of asthma and other chronic diseases (24), excessive physical activity may induce a stress reaction in the airways resulting in epithelial damage and inflammation (222). 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 (110). Elite athletes have a high risk to develop EIB, ranging from 30 to 70% of the athletes (94). Endurance sports and sports in combination to environmental factors such as chlorine or cold air are linked to the appearance of EIB (119, 223). Indeed, the 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 (189). 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 (93). Worldwide, different school programs 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 (101, 127).

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 (109, 224). It appears to be related to increased oxidative stress and inflammation in airways as well as systemic inflammation, even compromising sports performance (225, 226). 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 (94, 227, 228). The term air pollution includes particulate matter (PM) and gaseous compounds like ozone (O_3) (229). PM is categorized 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 (230), and McDonnell *et al* demonstrated a decrease in FEV_1 after exercise in exposure of O_3 compared with the same exercise performed in a filtered air environment (231).

Sputum induction is an important non-invasive tool of airway sampling (232). Furthermore, transcriptomic analysis on these sputum samples by next-generation sequencing (RNA-Seq) allows high-throughput and detailed characterisation of gene expression profiles (233). 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 hypothesize that the increased ventilation rate of adolescent elite athletes, in a strongly polluted area as Belgium, is associated with airway hyperreactivity and is reflected in their transcriptomic pattern.

3. Methods

Subjects

Early-career elite athletes of the 4 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 until December 2019. Control subjects, performing less than 6 hours of sport/week, were recruited to and included from January 2020 until March 2021 (Figure 1b). Athletes received one visit at their Elite Sports' Schools. The study visit of control subjects was performed in UZ Leuven.

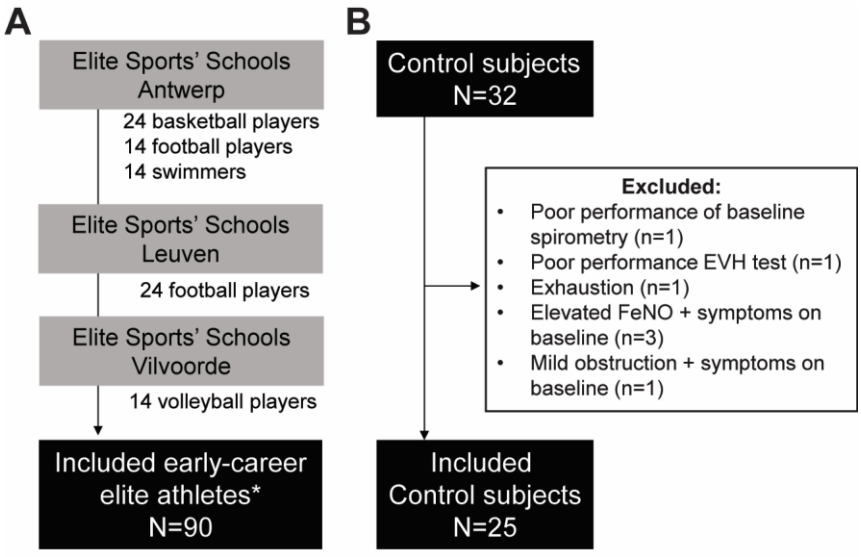

Figure 1: Recruited subjects. (A) Included athletes (n=90) in elite Sports' Schools in Antwerp, Leuven and Vilvoorde. * none were excluded. **(B)** Included controls **(n=25).** Exclusion criteria are shown.

Study design

The study design is presented in Figure 2. The study protocol was approved by the Ethical Committee of KU/UZ Leuven (S61602) and is registered on clinicaltrials.gov (NCT03587675).

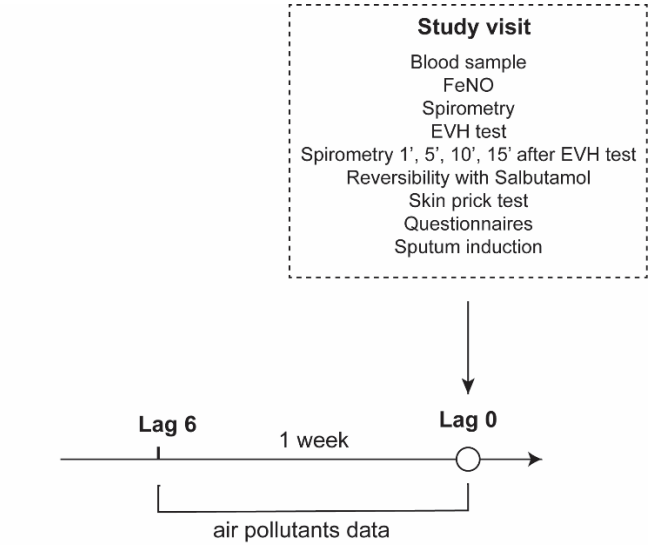

Figure 2: Study design. Demonstrating order of different interventions at the study visit. EVH, eucapnic voluntary hyperventilation.

FeNO

FeNO levels were measured with portable device "Niox Vero" (Accuramed, Belgium), recorded in parts per billion (ppb).

Spirometry & 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 ATS guidelines and adapted for this young age group (101, 130). Briefly, subjects inhaled a dry air mixture containing 5% CO_2 at room temperature for 6 minutes. The target ventilation to be maintained was 21xFEV_1, equivalent to 70% of MVV. The EVH test was considered to be positive if the fall in FEV_1 ≥ 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 9 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 (≥ 3mm and at least the size of the histamine control) (209).

Questionnaires

The AQUA© questionnaire (155, 156) as well as the asthma control test (ACT) (210) were filled in by all subjects. Additionally, a self-made exposure questionnaire, based on previous cohort studies (99, 101) was filled in to assess presence of airway symptoms (e.g. 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 minutes each. After inhalation, sputum was collected by spitting in a collection tube and processed by the selected plug method, to minimize contamination with saliva, like previously described (99, 182, 234–236). Sputum induction was not performed in swimmers, due to lack of consent in that particular sport branch. Cytospins (Shandon cytocentrifuge) were prepared from 12500 and 25000 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* (237). Briefly, digital images of 25 randomly selected airway macrophages from each cytospin slide were obtained at 1000x magnification. Cells were manually delineated and the ImageJ software (NIH, Maryland, US) automatically counted the number of particles and percentage area occupied by black carbon 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.(99, 182, 234–236) More information can be found in Supplementary 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 data supplement.

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 Supplementary 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 provides interpolated daily pollutant values in 4x4 km^2 grids from the Belgian telemetric air quality networks. Individual daily mean concentrations ($\mu g/m^3$) were calculated during a 7-day period prior EVH testing. The average outdoor temperature and humidity were obtained from "Royal Meteorological Institute of Belgium" (RMI). Environmental data were used to evaluate the association with response to EVH test. Exposure levels of each pollutant were divided into 3 subgroups based on data display IRCEL and WHO guidelines (2019): $PM_{2.5}$ low (<10 $\mu g/m^3$), immediate (10-25 $\mu g/m^3$) and high (>25 $\mu g/m^3$); PM_{10} low (<20 $\mu g/m^3$), immediate (20-50 $\mu g/m^3$) and high (>50 $\mu g/m^3$); O_3 low (<50 $\mu g/m^3$), immediate (50-100 $\mu g/m^3$) and high (>100 $\mu g/m^3$).

Statistical analysis

Statistical analysis was performed with Graphpad Prism 9 (Graphpad Software Inc, 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 ANOVA with Tukey's multiple comparisons test was used to compare a parameter between more than 2 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 Chi-square test. Correlation was studied by the Pearson or Spearman correlation test, depending on normality. IBM SPSS 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, 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% confidence intervals were transformed to [10^(B – 1) x 100]. This transformation allows interpreting the coefficient as the percentage of change in maximal fall in FEV_1. A difference was considered significant when $p<0.05$.

4. Results

Subject characteristics

Ninety adolescent elite athletes from 4 different sport disciplines were recruited: basketball (n=24), football (n=38), volleyball (n=14) and swimming (n=14) and 25 healthy controls between the age 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 to 21 hours/week. Baseline FEV_1% predicted of all athletes exceeded 80%, except for 1 football player previously diagnosed with asthma by physician having a FEV_1% of 69%. Significantly higher baseline FEV_1% and FVC% predicted values were observed in swimmers (mean ± SD: 121% ± 11) compared with all other sport disciplines and controls. In addition, thirty-three 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 poly-sensitized.

Table 1: Subject characteristics

				Sport disciplines			
	Control subjects	**Athletes**	**P-value**	Basketball players	Football players	Volleyball players	Swimmers
Number (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 (x 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 amongst controls and whole athletes' group. Normally distributed data are represented as mean ± SD and analysed via t-test. Non-parametric data are represented as median with interquartile range and analysed with Mann-Whitney test. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001, ****p-value < 0.0001 compared with control subjects.

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, 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%).

Black carbon 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, 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 black carbon were significantly increased in athletes compared with controls ($p<0.0004$) (Figure 3B). However, no significant difference was observed between in- and outdoor athletes (Figure 3C). One week and the past one-month average BC exposure did both not differ between controls and athletes (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).

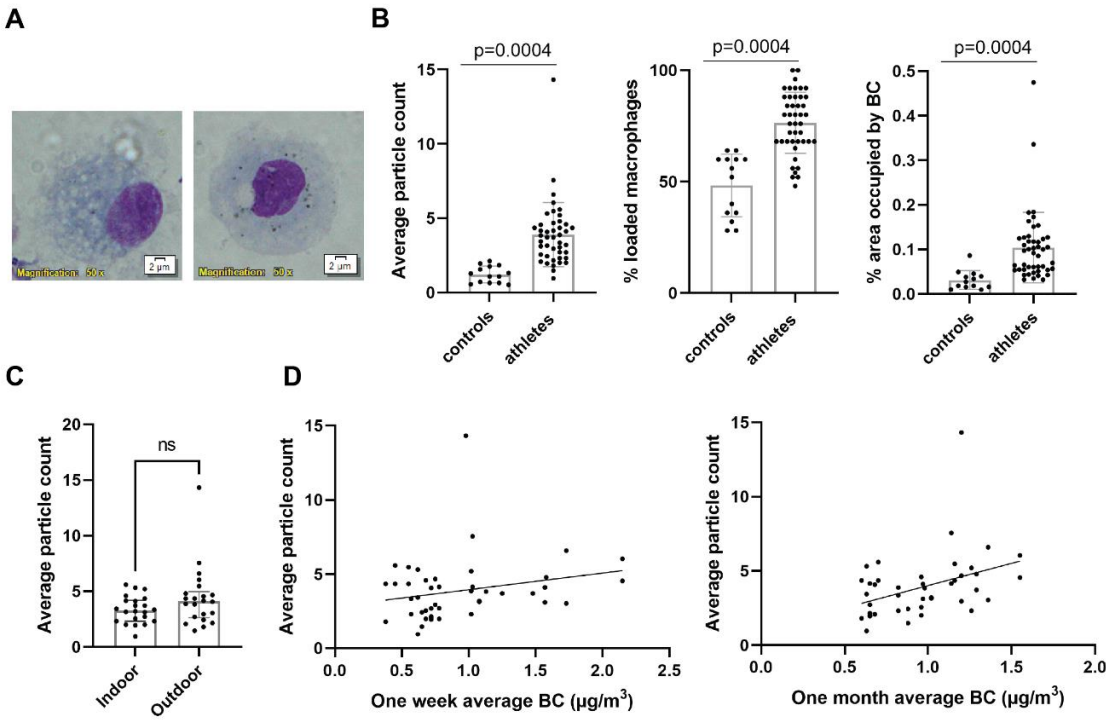

Figure 3: Increased carbon load in airway macrophages in athletes compared with controls. (A) Illustration of images captured for analysis showing airway macrophages stained by diff-Quick with increasing black carbon load. **(B)** The average particle count per macrophage, percentage of loaded macrophages, and the percentage area occupied by black carbon 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 black carbon load. (normality confirmed, unpaired t-test) **(D)** Correlation between one-week and one-month average BC (µg/m3) and the average particle count observed in athletes (n=44). (Spearman correlation)

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, Figure E1). 444 genes were differentially expressed between controls (n=11) and athletes (n=37) ($p<0.05$). 77 genes were upregulated and 367 genes were downregulated in athletes compared with controls. DEGs were mapped onto a volcano plot (Figure 4A, 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 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, 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 (Figure E4).

Damage-associated molecular pattern, including HMGB-1 and SpD, were 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).

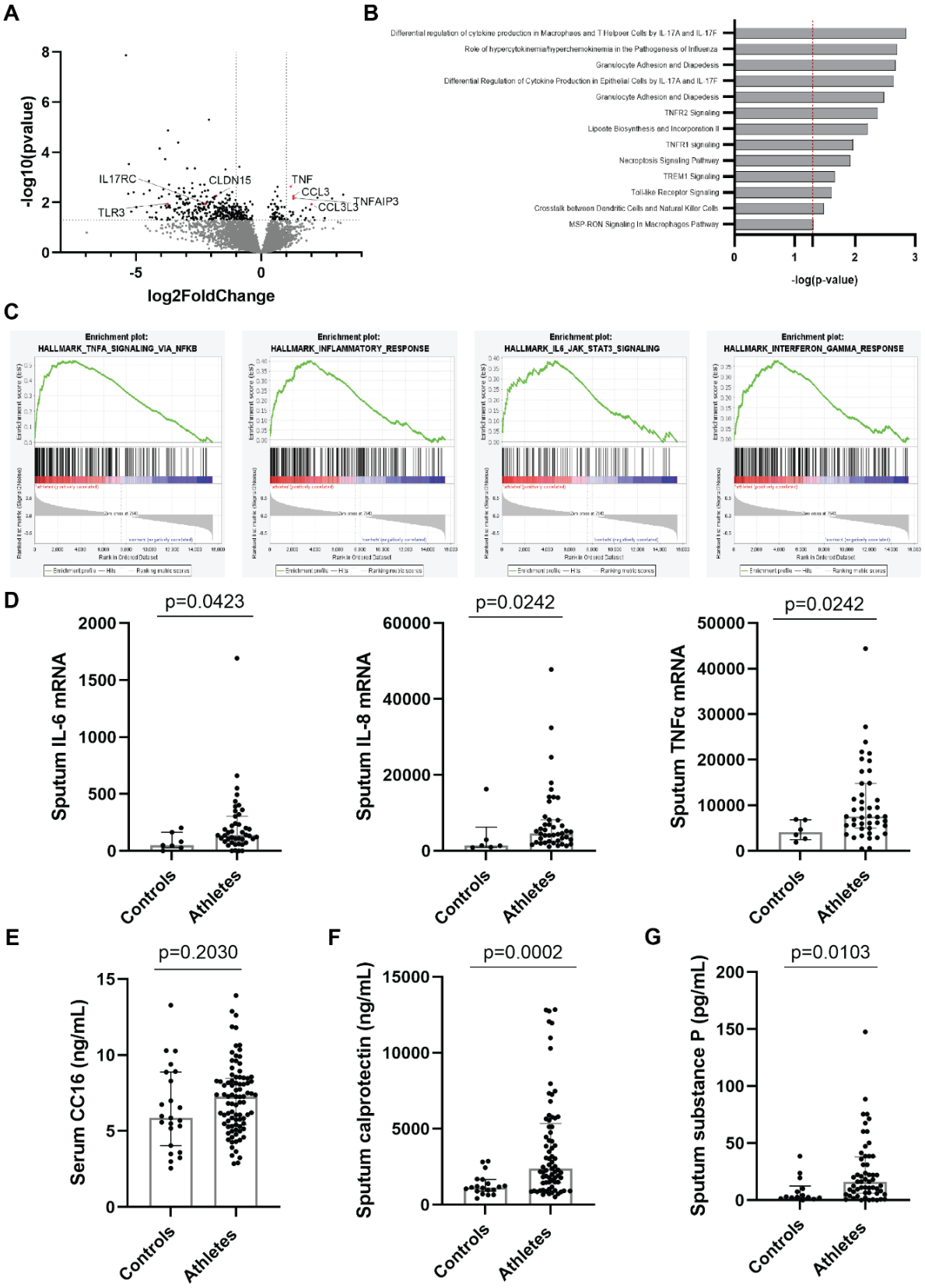

Figure 4: Profiling of serum and induced sputum samples in controls and athletes. (A) Volcano plot with the magnitude expressed as log2 fold change (x axis) and significance expressed as -log10 of the adjusted p value (y axis) of differential expression analysis. Genes of interest are labelled. **(B**) Selected significantly enriched or downregulated pathways based on IPA analysis listed according their p value. **(C)** Significant enrichment plots at 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 test)

EVH response in young elite athletes

Eight elite athletes (9%) tested positive for EIB (≥10% fall in FEV_1) (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 (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 7 athletes and 3 controls i.e. 10 to 25% fall in FEV_1 and was considered to be moderate (≥ 25-<50%) in one athlete. 9 subjects had their maximal fall in FEV_1 (%) under threshold of 10% within 10 minutes after the EVH test and two controls had a decline in FEV_1 later on, 15 minutes after the EVH test. Preliminary comparison between atopic EIB+ (n=2) and EIB- (n=4) athletes by RNA-Seq can be found in Supplementary (Figure E5, 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=0.0114, p=0.0147) (Figure 5D). The same trend is observed for CLDN1 mRNA levels (p=0.0579) (Figure 5D).

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 – -10.72	< 0.001
PM_{10}	-10.42	-10.21 – -10.67	< 0.001
O_3	-10.07	-9.93 – -10.21	0.289

Adjusted relative changes (%) with their 95% CI in maximal fall in FEV1

† : adjusted for humidity, temperature, age-squared, gender, BMI and atopic state.

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 – -10.38	0.163
PM_{10}	-11.27	-10.07– -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.

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 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 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 multi-pollutant model, considering both $PM_{2.5}$ and $PM_{10,}$ the association with PM_{10} appeared to be the most robust (table 3).

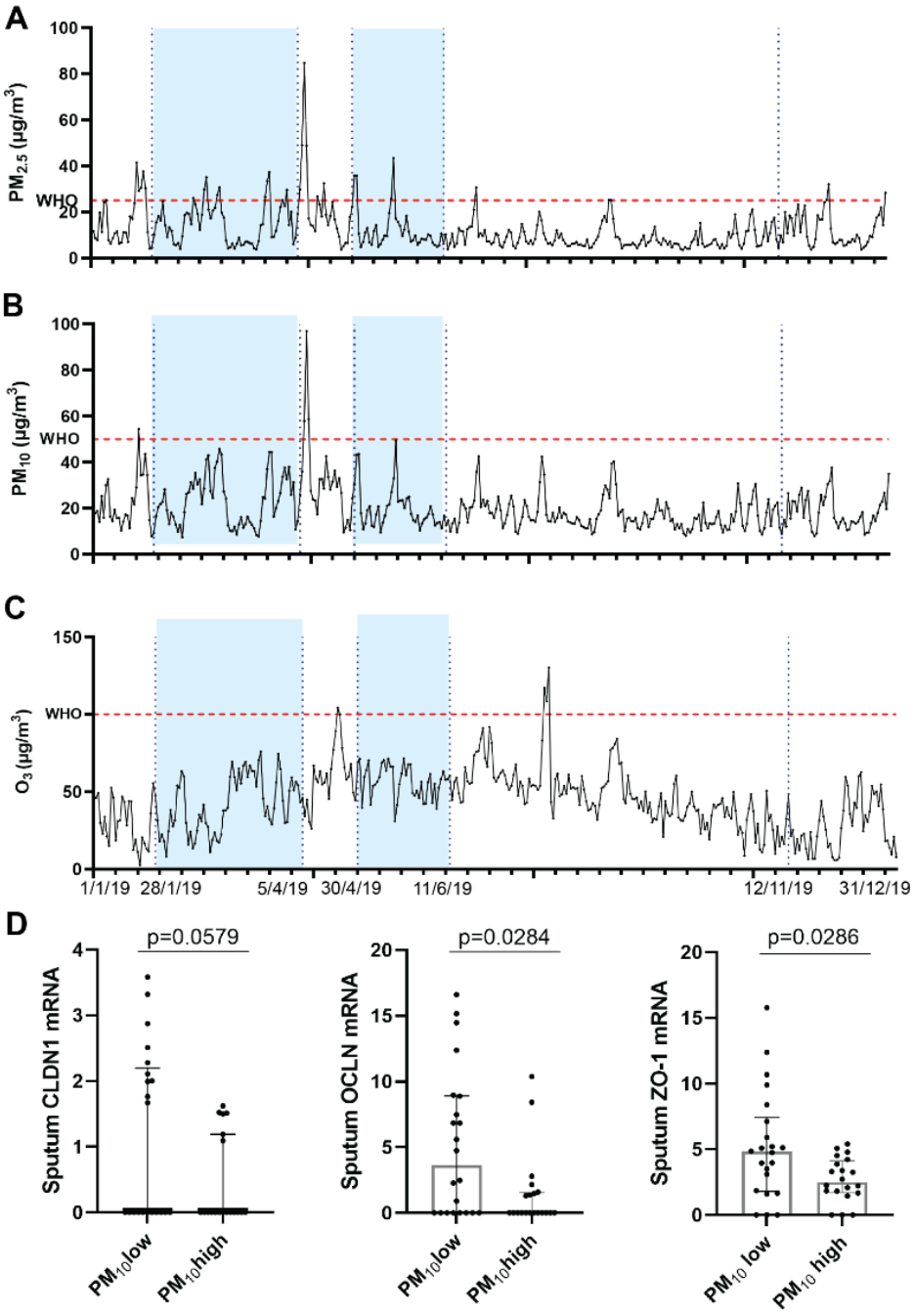

Figure 5: Air pollution exposure prior EVH test. (A, B, C) Daily mean levels of each pollutant considered during the study period averaged over the 3 included Elite Sport's Schools. The flashing red line refers to the maximum concentration of each pollutant established by WHO.(238) The dotted blue lines refer to the time frame the athletes participated in the study. **(D)** 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)

5. 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.

It is known that acute and/or chronic exposure to intense physical exercise can induce airway inflammatory reactions including cytokine release (239, 240). 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 (99, 241). However, few studies focus on local inflammatory markers in early-career athletes without asthma (205). 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 (99, 101, 104, 105), 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 downregulation 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* (184) 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 black carbon load (237, 242–244). Although the exact lifespan of human macrophages is unknown, $PM_{2.5}$ 6-month exposures are most strongly associated with airway macrophage black carbon content, rather than with shorter time periods considered (242, 245). We found a significant correlation between airway macrophages black carbon 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 to 6 days prior (246, 247). For the EVH test, such correlation was not described before. However, variability in the results of the EVH test has repeatedly been reported (131, 248, 249). The algorithm for the evaluation of asthma and EIB in athletes by Boulet *et al* 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 (100). This environmental condition might very well be PM exposure. In addition, the recent position paper of European Academy of Allergy and Clinical Immunology (EAACI) highlights the importance of repeat assessment and requirement of in season testing (202).

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 (250, 251). 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 (252). In line with previous studies, we found a downregulation of TLR3 in athletes compared with controls (253, 254). This may contribute to the higher reported susceptibility for infections in athletes (253). 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 co-receptor to respond to IL-17A and IL-17F (255). 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 upon 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 nonatopic 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 hyperreactivity.

6. Supplementary

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 (99, 182, 234–236). Data was normalized to the geometric mean of the reference genes PPIA and RPL13A, determined with RefFinder (256). 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 (257).

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(258). 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 (259). 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 (260). 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.

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.(99, 101) 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 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 (261). Elite sport activities already have been described as stimulus able to induce oxidative stress (262), 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 (93). 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

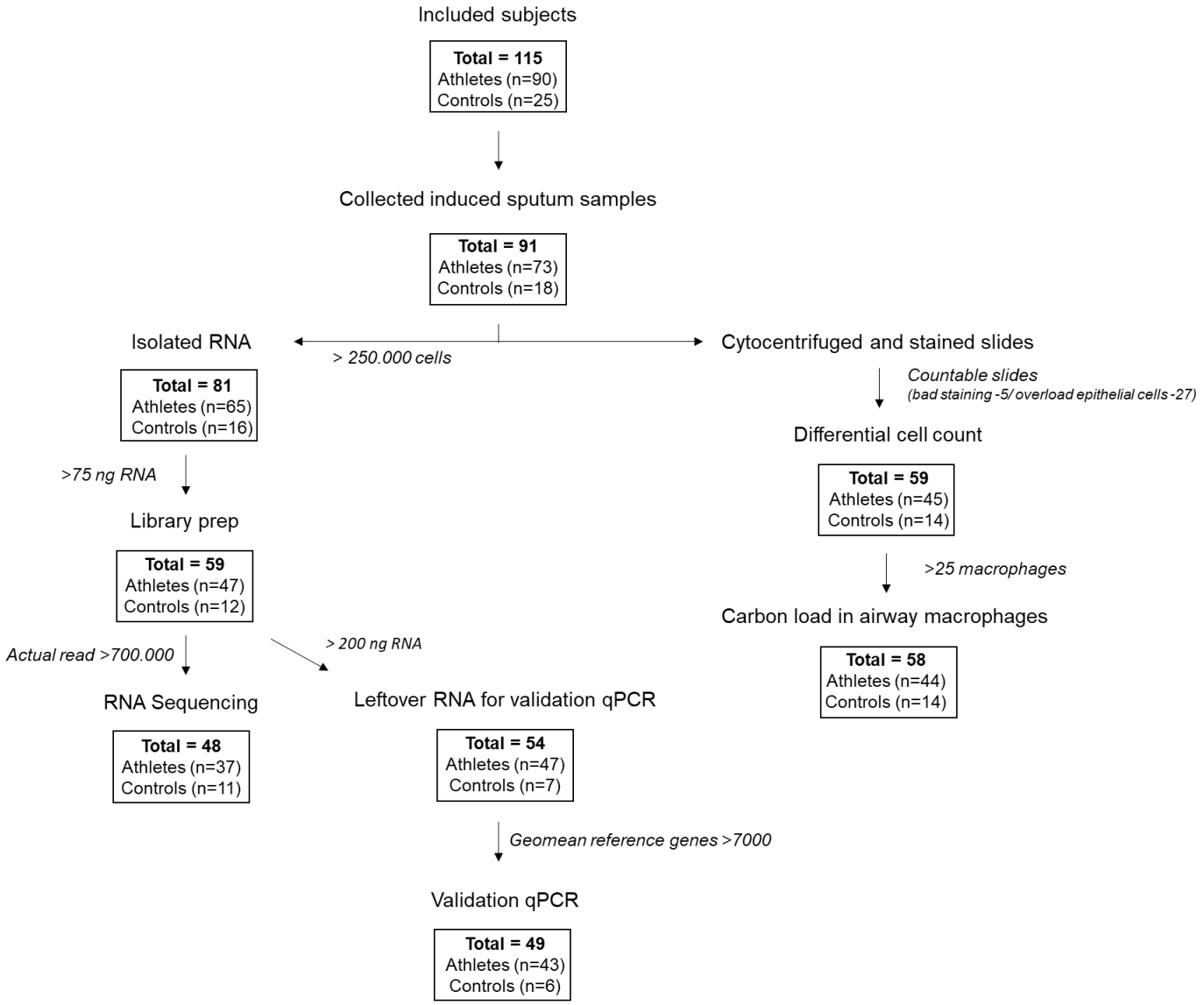

Figure E1: Flow diagram sputum sampling

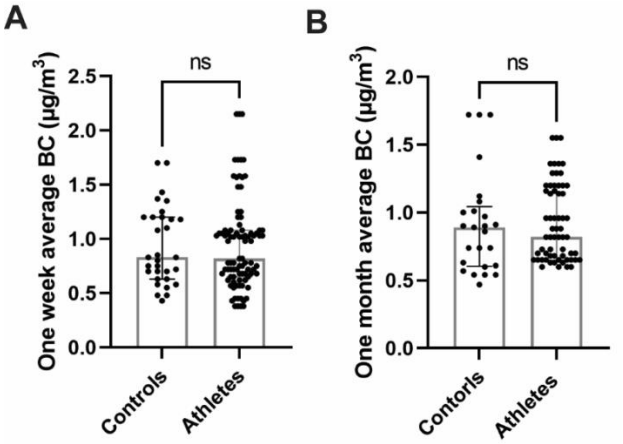

Figure E2: One-week and one-month average BC exposure in controls vs athletes.
Comparison of one week **(A)** and one-month **(B)** average BC (µg/ m3) between controls and athletes. (Mann-Whitney test)

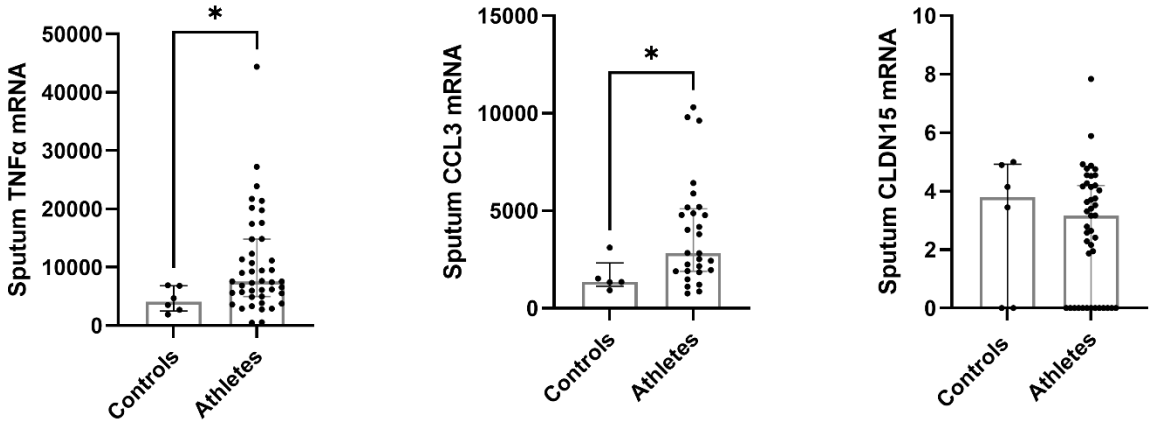

Figure E3: Validation qPCR controls vs athletes Remaining RNA for controls (n=6) and athletes (n=43) was used to validate obtained results via qPCR. (Mann-Whitney test) *p<0.05.

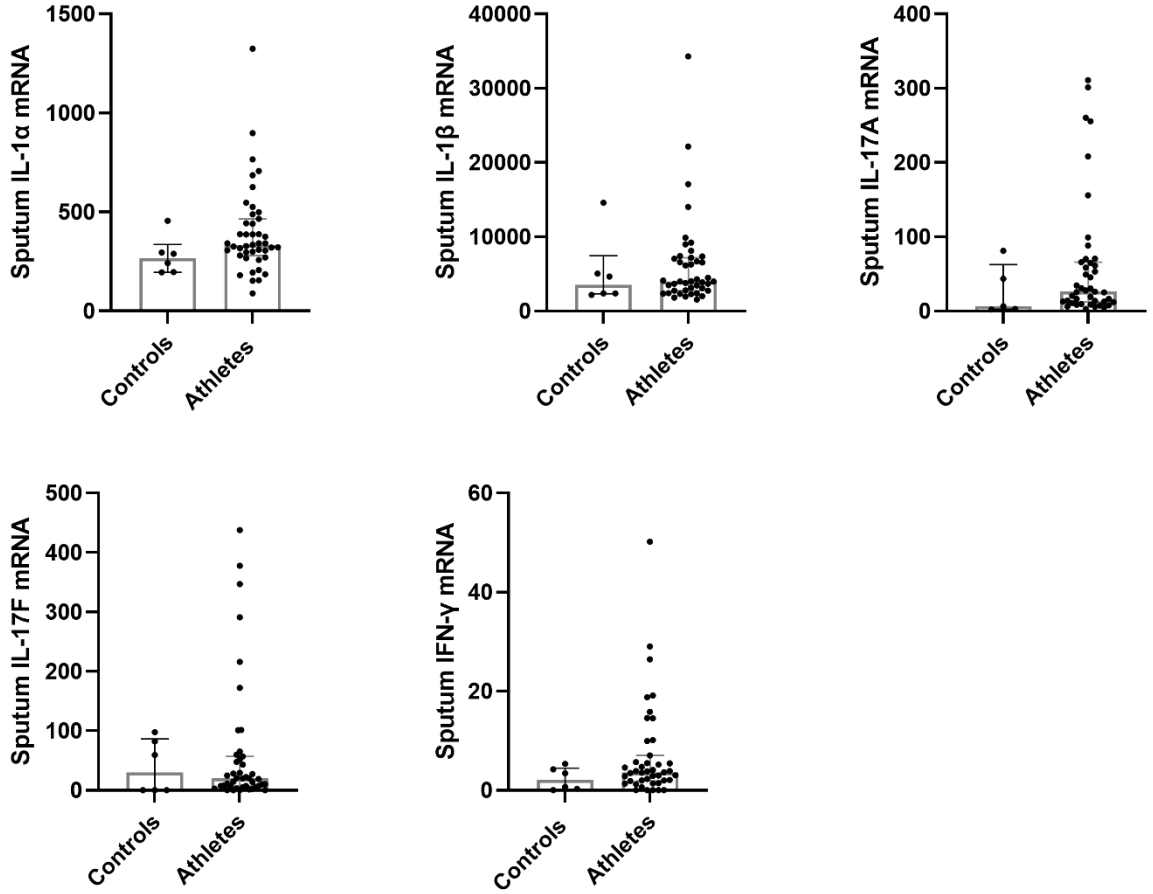

Figure E4: qPCR cytokines controls vs athletes
Remaining RNA for controls (n=6) and athletes (n=43) was used to study the cytokine profile in controls vs athletes via qPCR. (Mann-Whitney test)

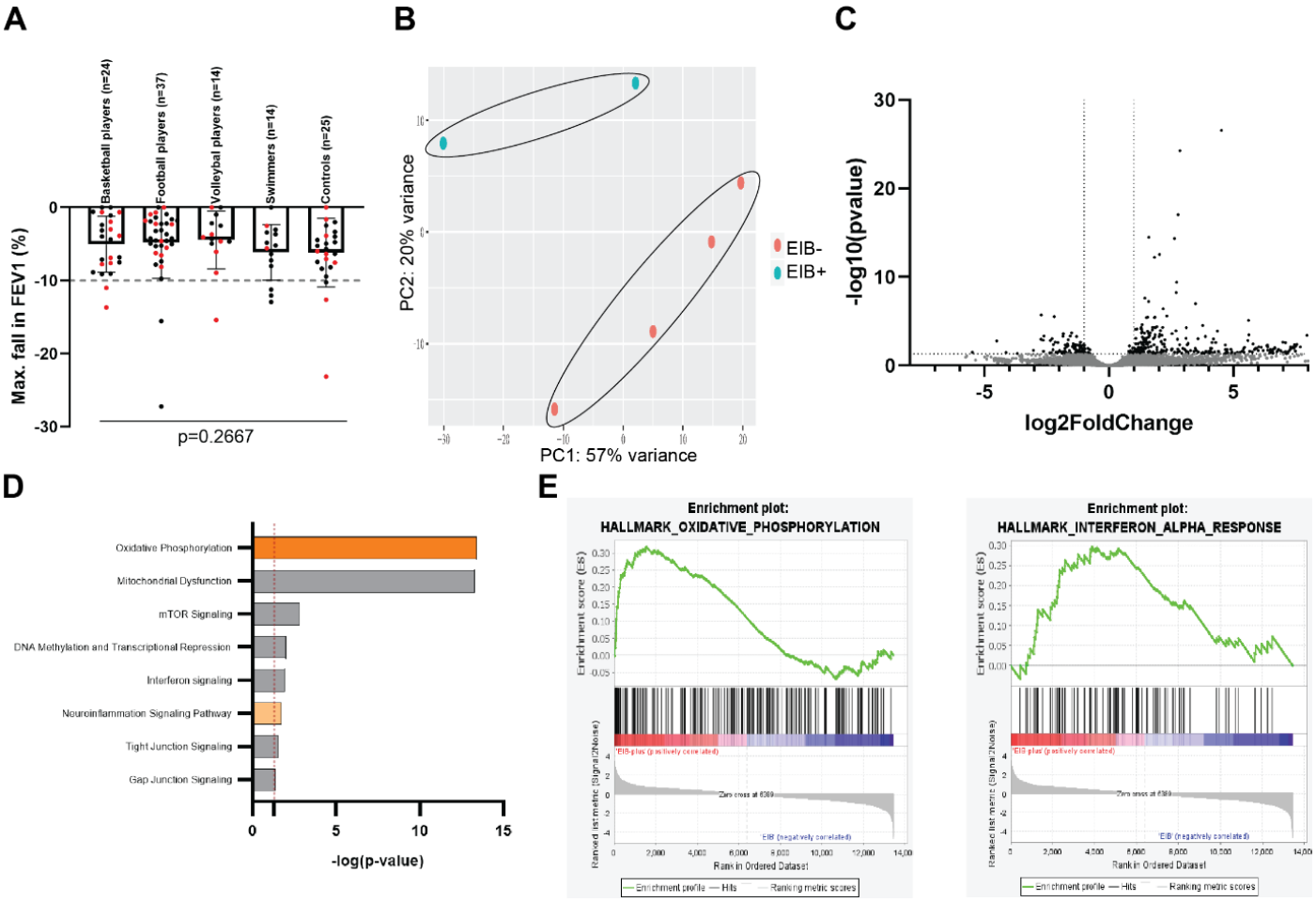

Figure E5: Transcriptome profiling of induced sputum samples in EIB- and EIB+ athletes. (A) EVH test was completely performed in basketball players (n=24), football players (n=37), volleyball players (n=14), swimmers (n=14) and control subjects (n=25). The test was considered positive if a drop in FEV ≥ 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%.

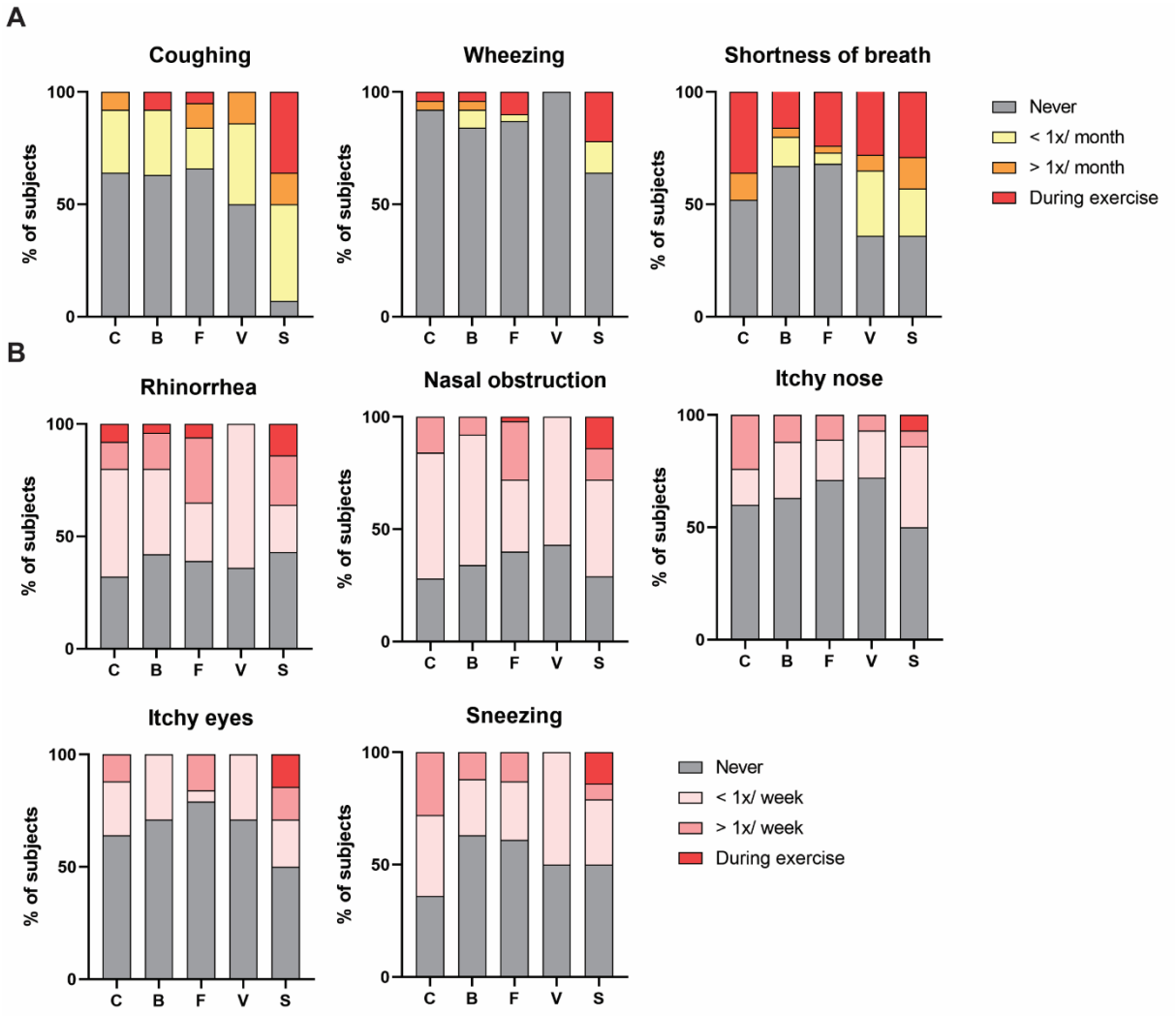

Figure E6: Self-reported upper and lower airway symptoms
Reported symptoms in lower (A) and upper airways (B) were evaluated via a symptom questionnaire in controls ('C', n=25) basketball ('B', n=24), football ('F', n=38), volleyball players ('V', n=14) and swimmers ('S', n=14). Data are expressed as 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).

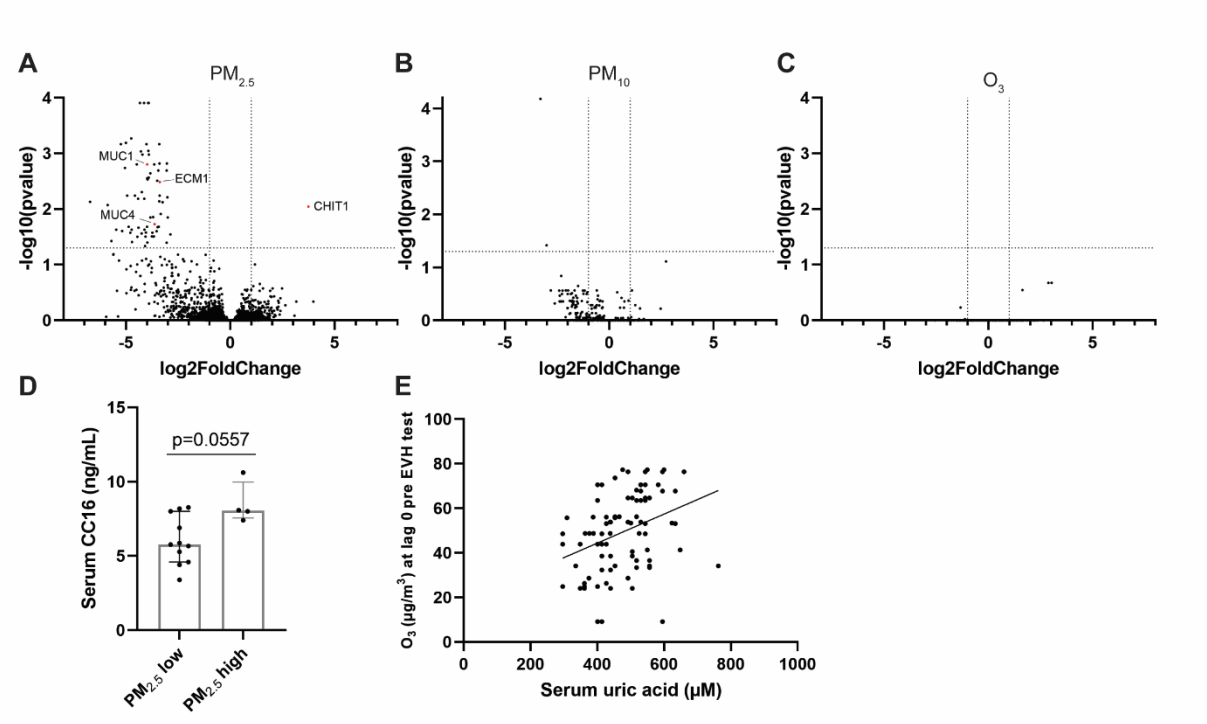

Figure E7: Transcriptomic differences in athletes based on air pollution exposure.
Volcano plot with the magnitude expressed as log2 fold change (x axis) and significance expressed as -log10 of the adjusted p value (y axis) of differential expression analysis for $PM_{2.5}$ low (<10 µg/m³) vs high (>25 µg/m³) (A), PM_{10} low (<20 µg/m³) vs immediate (>20 µg/m³) (B) (no values above threshold of WHO (50 µg/m³)) and O_3 low (<25 µg/m³) vs immediate (O_3>50 µg/m³) (C) (no values above threshold of 100 µg/m³), respectively. (D) Serum CC16 levels in athletes exposed to low $PM_{2.5}$ (<10 µg/m³) compared to high $PM_{2.5}$ (>25 µg/m³) levels. (Mann-Whitney test). (E) Correlation of serum uric acid levels with O_3 levels at the day of study visit. (spearman correlation).

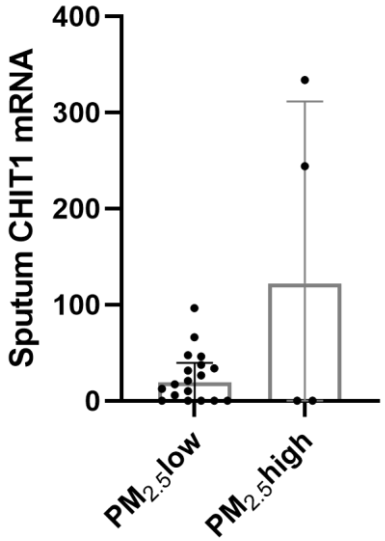

Figure E8: Validation qPCR CHIT1
Remaining RNA for controls athletes exposed to low PM2.5 levels (<10 µg/m³) (n=18) and PM2.5 high levels (>25 µg/m³) (n=4) was used to validate obtained results via qPCR. (Mann-whitney test).

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

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

Chapter 7

Epithelial damage and exposure to irritants in asthmatics

Manuscript in preparation

Janne Goossens*, Anne-Charlotte Jonckheere*, Sien De Boodt, Ellen Dilissen, Nora Marain, Lieven Dupont, Sven Seys, Dominique Bullens

*Contributed equally

Part of: Validation of cluster analysis of sputum cytokine profiles in asthmatic patients reveals underlying molecular pathways.

1. Introduction

Asthma is a very heterogeneous disease in which several pheno- and endotypes can be distinguished based on allergic status, inflammatory pattern, severity, age of onset or the presence or absence of a Type 2 profile (8, 263). It is suggested that the airway epithelium is crucial in pathophysiology of asthma (264). The airway epithelium is the first barrier for external triggers.

It has been reported that certain environmental exposures, such as exposure to cigarette smoke and occupational triggers, may alter the type of immune response and cytokine production (265, 266). Moreover, asthma is associated with different comorbidities, such as allergic rhinitis, chronic rhinosinusitis (CRS) with nasal polys, obesity, obstructive sleep apnoea (OSAS) and gastroesophageal reflux disease (GERD) that complicate disease management (267). These comorbidities could also affect airway inflammation trough upregulation of adhesion molecules and inflammatory cells and systemic inflammation (268).

In this thesis, we investigated the overlap of comorbidities in asthma. Furthermore, we focused on the effect of environmental triggers on the sputum transcriptome of asthmatic patients. We hypothesized that environmental triggers such as cigarette smoke and work-related exposures impact the immunological response in patients with asthma, in which epithelial damage plays an important role.

2. Methods

Study design

A cross-sectional cohort study was designed with a retrospective analysis of patients' files and a new analysis of sputum mRNA levels and sputum supernatant molecules from stored sputum samples of asthma patients compared to healthy controls was performed (see supplementary methods). All participants provided written informed consent and the Ethical Committee Research UZ/KU Leuven approved the study (S52549).

Clinical characteristics

All comorbidities were doctor-diagnosed which means that the diagnosis was made by the attending physician and noted in the patient medical file. If no entries were made, we assumed that the comorbidity was not present. Seven comorbidities were studied: CRS, functional endoscopic sinus surgery (FESS), GERD with active symptoms, obesity (BMI ≥ 30 kg/m2),

asthma COPD overlap syndrome (ACOS), allergy and OSAS. Allergy was defined based on a history or anamnesis of doctor-diagnosed allergy. Additional information can be found in the supplementary methods.

Sputum induction and analysis

The left-over sputum samples were provided by the Clinical Laboratory (LAG) of UZ Leuven. The samples were processed by LAG via the selected plug method as described by Pizzichini *et al.* (269) into a single cell suspension and sputum differential cell count was determined. The remaining cells and sputum supernatant were transferred to the Allergy and Clinical Immunology Research Group (LACI) and the newly induced sputum samples of healthy controls were processed and cells were stored in RNA lysis buffer (Qiagen, Westburg) together with the sputum supernatant in the -80°C.

Bulk RNA sequencing

Sixty-seven sputum samples had high enough quality to perform bulk RNA-seq. RNA libraries were constructed using QuantSeq 3' mRNA library prep kit (Lexogen, Vienna, Austria). Sequencing was performed using the HiSeq 4000 (Illumina, San Diego, USA). Up- and down-regulated DEGs were identified by a log2 fold change ≥1 or ≤ −1 and a false discovery rate adjusted p value of ≤0.05. A detailed description on library preparation, bioinformatics processing and differential gene expression analysis is available in data supplement.

Analysis of molecules in sputum supernatant

Details on the markers determined in the sputum supernatant according to manufacturer's instructions are described in table 1.

Table 1: Assays used for analysis sputum supernatants.

	Asthmatic patients (n=)	Controls (n=)	Kit	Manufacturer	Dilution
Uric acid	96	28	AmplexTM Red Uric Acid/Uricase Assay kit (#A22181)	Thermo Fisher, Waltham, USA	1/5
SP-D	96	28	Human DuoSet ELISA (#DY1920)	R&D Systems, Minneapolis, USZ	undiluted
HMGB1	94	28	Human HMGB1 ELISA (#abx351796)	Abbexa, Cambrige, UK	undiluted
Substance P	90	28	Substance P ELISA (#583751)	Cayman Chemical, Michigan, USA	1/2

Calprotectin	90	28	Calprolab Calprtectin ELISA (ALP, #CALP0170)	CALPRO AS, Lysaker, Norway	1/20

SP-D, Surfactant protein D; HMGB1, High mobility group box 1

Sputum cytokine profiles in asthma patients: cluster analysis

Real time qPCR (BioRad, Hercules, USA) was performed for mRNA of IL-17F and IL-22, normalized to the reference gene β-actin (182, 236). Sputum cytokine mRNA 10th and 90th percentile values of expression levels in healthy subjects were calculated to determine lower and upper cut-off levels for the various cytokines measured by qPCR (table 1).

Table 2: mRNA cytokine levels in asthmatic patients and healthy controls and defined cut-off values.

	Asthmatic patients (n = 73)	**Controls (n= 34)**	**P-value**
Sputum IL-17F mRNA	42.2 (3.08 – 241.5)	8.03 (0 – **59.4**)	< 0.0001
Sputum IL-22 mRNA	0 (0 – 0.5)	0 (0 – **0.3**)	0.883

Data are represented as median with 10 and 90% percentiles between brackets. P-value was determined with Mann-Whitney test. NA = not applicable. Defined cut-off values based on 90th percentile are indicated in bold.

Statistical analysis

Statistical analysis was performed using GraphPad Prism v.9 for Windows (La Jolla, CA, USA). Normal distribution was studied for each data set with a D'Agostino & Pearson normality test. One-way ANOVA with Tukey post-hoc test or Kruskal-Wallis test with Dunn's multiple comparison test was used where appropriate. Mann-Whitney test or unpaired t-test was used to compare two groups where appropriate. Bonferroni was applied to correct for multiple testing. A difference was considered significant when $p < 0.05$.

3. Results

Patient characteristics

Hundred and three asthmatic patients and 34 healthy control subjects were included in this study (table 3). Smoking was more present in the asthmatic patients compared to healthy controls where almost all subjects were non-smokers (table 2). In addition, allergies were significantly more present in asthmatic patients (67%) compared to healthy controls (36%). Most asthmatic subjects were allergic to house dust mite (49.5%), grass pollen (38.9%), tree pollen (36.8%) and animals (37.9%) such as cat, dogs, rabbit and horses. Looking at the different comorbidities, 29% of asthma patients had underlying CRS of which 18% received a FESS. GERD occurred in 18% of the asthmatic patients. Twenty-three percent of asthmatic patients were obese, while none of the healthy control subjects had a BMI ≥ 30 kg/m^2. Eleven asthmatic patients had ACOS (10.6%) and 10 patients OSAS (10%). Figure 1 summarizes the overlap and interactions of the different comorbidities in patients with asthma.

Table 3: Subject characteristics

	Asthmatic patients	Controls	P-value
Subjects (n=)	103	34	
Age (years)	48 (35 – 57)	33 (28.8 – 45.8)	0.002
Gender (M/F)	42/61	20/14	0.076
Body Mass Index (kg/m^2)	26.1 (23.1 – 29.6)	23.4 (21.8 – 24.5)	0.009
Smoking (active/ex-/non-)	15/32/27	1/3/29	< 0.0001
Allergy (%)	69 (67%)	12 (36%)	0.0004
Inhaled steroids only (%)	69 (67%)	NA	NA
Inhaled and oral steroids (%)	18 (17%)	NA	NA
FEV_1 % predicted (%)	87.5 ± 20.3	107.5 ± 13	< 0.0001
FEV_1/FVC (%)	72.7 (62.3 – 79.8)	78.7 (75.9 – 82.7)	0.0002
Sputum eosinophils (%)	1 (0 – 9.7)	0 (0 – 1)	0.0002
Sputum neutrophils (%)	59.5 (39 – 78.5)	23.5 (10 – 42.6)	< 0.0001
Sputum macrophages (%)	25.8 (13 – 44.5)	75.5 (56.2 – 90)	< 0.0001
Sputum lymphocytes (%)	2 (1 – 3.4)	0 (0 – 0)	< 0.0001
Number of samples for RNA-Seq	67	0	NA

Normally distributed data are represented as mean with standard deviation and unpaired t-test was used to determine P-value. Not normally distributed data are represented as median with 25 and 75% percentiles between brackets and Mann-Whitney test was used to determine P-value. NA = not applicable

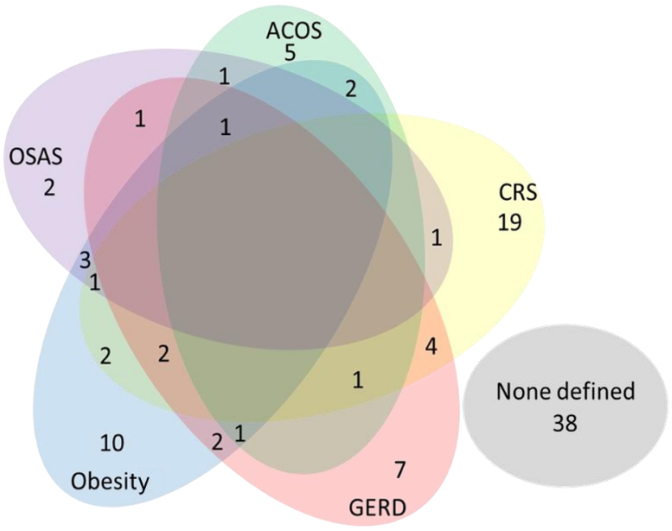

Figure 1: Venn diagram of the comorbidities and their interactions in patients with asthma. Asthma-COPD overlap syndrome (ACOS), chronic rhinosinusitis (CRS), Gastroesophageal reflux disease (GERD), obstructive sleep apnoea syndrome (OSAS).

Airway inflammation in asthmatic patients

The percentage of eosinophils significantly and negatively correlated with the lung function (FEV_1(%) and TI(%)) of patients with asthma (table E1). Several damage-associated molecular patterns (DAMPs), such as uric acid, SP-D and HMGB-1, calprotectin (a marker for neutrophilic inflammation) and substance P (a marker for neurogenic inflammation) were studied in our patient cohort compared to healthy controls. DAMPs were significantly increased in sputum supernatant of asthmatic patients compared to healthy control subjects (figure 2A). In addition, significantly higher levels of calprotectin were measured in sputum supernatant of asthmatic patients compared to control subjects (figure 2A). As most of the asthmatic patients had underlying neutrophilic inflammation, this finding was not unexpected. This was further confirmed by a significantly positive correlation between calprotectin and sputum neutrophils in asthmatic patients (Spearman $r=0.444$, $p<0.0001$, data not shown). Calprotectin levels were significantly increased in neutrophilic asthma patients compared to eosinophilic and pauci-granulocytic asthma patients (figure 2B). Furthermore, sputum calprotectin levels and also HMGB-1 and SP-D significantly and negatively correlated with FEV_1(%) (table E1). In contrast, no significant differences were found in DAMPs and substance P levels in sputum supernatant amongst inflammatory phenotypes (figure 2B).

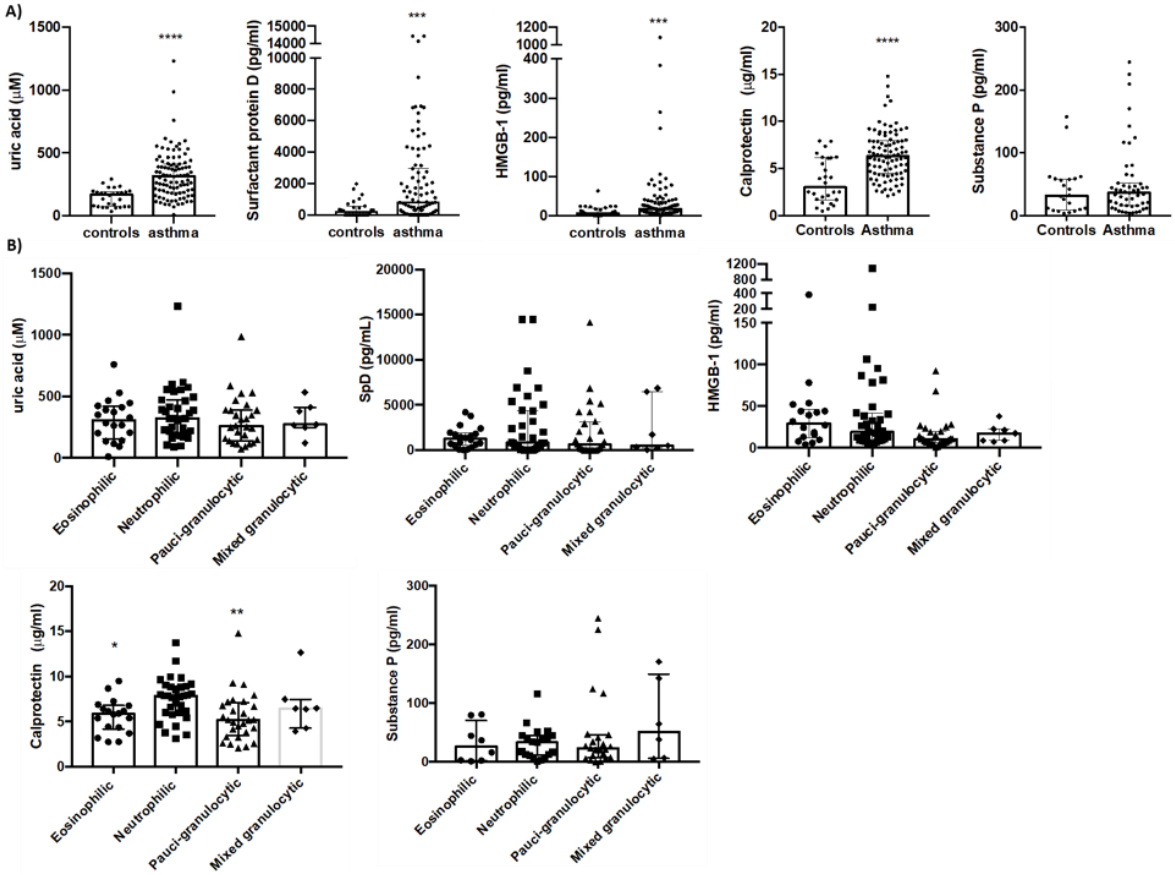

Figure 2*:* **Protein levels measured in sputum supernatant of controls and patients with asthma.** Following mediators were determined in sputum supernatant of controls and patients with asthma **(A)** and compared amongst inflammatory phenotype **(B)**: Uric acid, Surfactant protein D, HMGB-1, Calprotectin, Substance P. Data is presented as median with IQR. Mann-Whitney test was performed. *p < 0.05, **p < 0.01, ***p < 0.001. Kruskal-Wallis test was performed with Dunn's multiple comparisons test. *p<0.05 Eosinophilic vs neutrophilic **p < 0.01 Neutrophilic vs pauci-granulocytic.

Airway damage based on exposure in asthmatic patients

An important exposure that influences airway inflammation is smoking. Never (n=13), former (n=20) and active (n=8) smokers were analyzed by RNA-seq. 90 DEGs (22 up- and 68 downregulated) were observed in never versus active smokers (figure 3A). Cytochrome P450 1B1 (CYP1B1) was clearly upregulated, while CLDN4, ECM1 and TJP1 were downregulated in active compared to never smoking asthmatic patients (table E2). Besides, 15 DEGs (6 up- and 9 downregulated) were observed when comparing former versus active smokers (figure 3A, table E3). CYP1B1 was upregulated, which is involved in the aryl hydrocarbon receptor (AhR) and xenobiotic metabolism signalling and nicotine degradation (figure 3B). Even the comparison between never and former smokers resulted in 64 DEGs (table E4), which were all downregulated in former smoking asthmatic patients (figure 3A). Those DEGs were related to epithelium development and filament cytoskeleton organization (figure 3C).

In addition, the presence of work-related exposures to irritants was examined. Only exposures with an occurrence over 5 patients in the cohort were included. In total 7 patients with asthma reported work related exposure to cleaning products. Sputum SP-D levels were significantly elevated in patients with asthma who reported exposure to cleaning products compared with patients who did not report any occupational exposure (figure 3D). We found 6 significant DEGs between the group of patients with asthma who reported exposure to cleaning products (n=6) and who did not report exposure (n=14) (1 up- and 5 downregulated, figure 3E, table E5). IPA analysis also here revealed the role of AhR signalling, which is known to be a sensor of xenobiotic chemicals (Figure 3F). Regarding the downregulated genes, noise was identified as the top downregulated gene is Y-chromosome gene, as all of the exposed asthmatic patients to cleaning products were female. The expression of IL-17 and IL-22 was investigated in association with asthmatic patients exposed to cleaning products, as it is known that AhR regulates IL-17 and IL-22 production. A significant association was found for asthmatics with work related exposure to cleaning products and having an IL-22 high cytokine profile ($p=0.0371$). For IL-17 no significant association was found ($p= 0.0892$), even though none of the asthmatics with work related exposure to cleaning products had an IL-17 high cytokine profile. Only the occupation “nurse” appeared also > 5 times (n=7) but did not show any DEG compared to patients with asthma who did not report any occupational exposure.

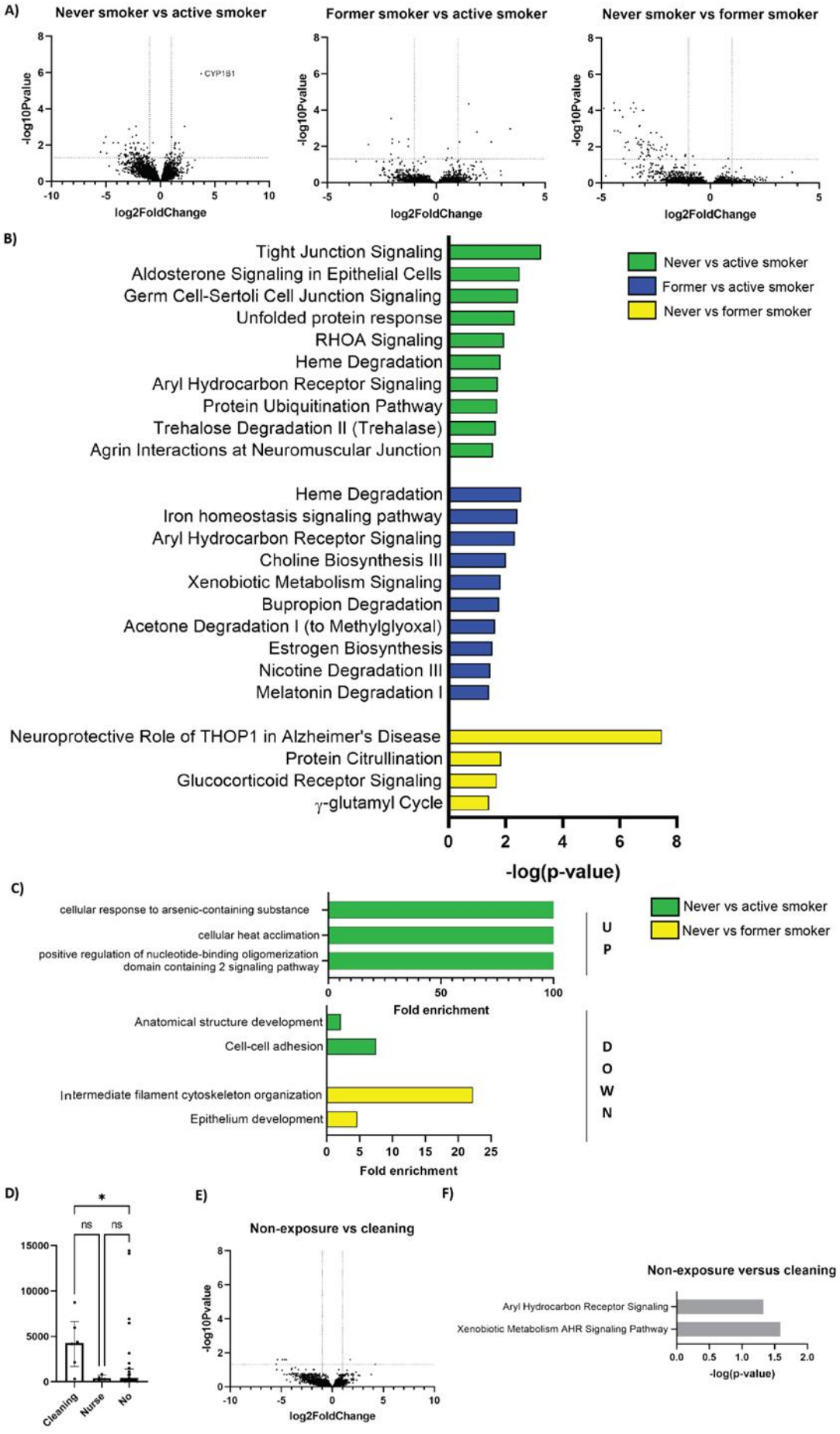
A)
Never smoker vs active smoker
Former smoker vs active smoker
Never smoker vs former smoker
CYP1B1
-log10Pvalue
log2FoldChange
B)
Never vs active smoker
Former vs active smoker
Never vs former smoker
Tight Junction Signaling
Aldosterone Signaling in Epithelial Cells
Germ Cell-Sertoli Cell Junction Signaling
Unfolded protein response
RHOA Signaling
Heme Degradation
Aryl Hydrocarbon Receptor Signaling
Protein Ubiquitination Pathway
Trehalose Degradation II (Trehalase)
Agrin Interactions at Neuromuscular Junction
Heme Degradation
Iron homeostasis signaling pathway
Aryl Hydrocarbon Receptor Signaling
Choline Biosynthesis III
Xenobiotic Metabolism Signaling
Bupropion Degradation
Acetone Degradation I (to Methylglyoxal)
Estrogen Biosynthesis
Nicotine Degradation III
Melatonin Degradation I
Neuroprotective Role of THOP1 in Alzheimer's Disease
Protein Citrullination
Glucocorticoid Receptor Signaling
γ-glutamyl Cycle
-log(p-value)
C)
Never vs active smoker
Never vs former smoker
cellular response to arsenic-containing substance
cellular heat acclimation
positive regulation of nucleotide-binding oligomerization domain containing 2 signaling pathway
UP
Fold enrichment
Anatomical structure development
Cell-cell adhesion
Intermediate filament cytoskeleton organization
Epithelium development
DOWN
Fold enrichment
D)
ns
ns
*
Cleaning
Nurse
No
E)
Non-exposure vs cleaning
-log10Pvalue
log2FoldChange
F)
Non-exposure versus cleaning
Aryl Hydrocarbon Receptor Signaling
Xenobiotic Metabolism AHR Signaling Pathway
-log(p-value)

Figure 3: Gene expression in induced sputum samples of patients with asthma upon exposures. A) Volcano plot showing Log2FoldChange and statistical significance of individuals genes of sputum of patients with asthma who never smoked compared to active smokers, who smoked > 4 weeks ago (former smoker) compared to active smokers and never smoked compared to who smoked > 4 weeks ago (former smoker). **B)** IPA analysis of differentially expressed genes (DEGs) (p value < 0.05, Log2FoldChange < -1 and >1) for never versus active smokers (green), former versus active smokers (blue) and never versus former smoker (yellow). **C)** GO analysis showing enriched GO terms for upregulated DEGs in the active smokers compared to never smokers and associated GO terms for downregulated DEGs of the active smokers versus the never smokers and for the former smokers versus the never smokers. **D)** Surfactant protein D levels in sputum supernatant of patients with asthma reporting work related exposure to cleaning products (n=6), reporting nurse (n=4) and those who did not report any occupational exposure (n=28). Kruskal-Wallis test with Dunn's multiple comparisons test was performed. *p < 0.05. **E)** Volcano plot showing Log2FoldChange and statistical significance of individuals genes of sputum of patients with asthma without occupational exposure compared to patients with cleaning exposure. **F)** Ingenuity Pathway Analysis (IPA) of differentially expressed genes (DEGs) (p value < 0.05, Log2FoldChange < -1 and >1). The grey bars indicate the -log(p-value). No z-scores were available.

4. Discussion

In this study, we demonstrated increased epithelial cell damage in asthmatic patients compared to healthy controls, which was expressed as increased levels of damage-associated molecular patterns, such as uric acid, SP-D and HMGB-1. This is proposed to play an important role pathogenesis of asthma (264). In addition, the airway epithelium plays a key role in the response to environmental triggers. We indeed found significant differences amongst sputum transcriptome exposed to cigarette smoke and work-related exposure to cleaning products compared with asthmatic patients without exposure. The AhR pathway was found to play a role in airway inflammation in asthmatic patients exposed to irritants.

Increased work-related exposure to cleaning products was associated with airway damage (increased SP-D levels), and the involvement of AhR signalling pathway. Furthermore, an association with an 'IL-22-high' cytokine profile was found for asthmatics exposed to cleaning products. AhR, which is linked to exposure to cleaning products in our patients, is pivotal for IL-22 production by different cell types (19). In addition, AhR has been connected to the regulation of asthma phenotypes in a murine model of occupation/irritant-induced asthma (18) and in a tobacco smoke-induced asthma model (19) suggesting that AhR modulates airway inflammation in response to specific chemical agents (18).

Available evidence suggests that cleaning materials can exacerbate as well as induce the development of asthma trough irritant and immunological mechanisms (270, 271). Aryl hydrocarbon receptor repressor (AHRR) is known to be a competitive repressor of AhR activity (272). AHRR was significantly upregulated in the group of patients with exposure to cleaning products compared to patients without exposure. AhR is recognized to be a key regulator of homeostatic processes at barrier sites such as skin, lung and intestines and is broadly expressed in immune cells and non-hematopoietic cells (273). Its activation induces ILC3

maintenance and regulates IL-17 and IL-22 production (274). However, AhR may also play a protective role on lung inflammation after cigarette smoke (275). Studies have shown that activation of AhR can promote the development of regulatory T cells and reduce the number of Th17 cells (276). In our cohort, asthmatics exposed to cleaning products were associated with IL-22 high cytokine profile, while none of those asthmatics had high IL-17 cytokine levels. The nature of the ligands determine the type of immune response induced by AhR activation (277).

Cigarette smoke, a complex chemical mixture, is known as an environmental asthma trigger. Smoking induced downregulation of multiple tight junctions compared to non-smokers in asthma patients, like described by others *in vitro* or COPD patients (278, 279). CYP1B1 was significantly upregulated. CYP1B1 is a part of the cytochrome P450 family and metabolizes tobacco smoke components (280). This gene has already been correlated to active smoking and the interaction between early-life exposure to smoking and childhood asthma (280). In a recent sputum proteomics study, current and ex-smokers with asthma were compared and similarly found increased xenobiotic metabolism (CYP1B and ALDH3A1) (281).

Asthma exists with numerous comorbidities that can influence its clinical expression (268). Moreover, it has been demonstrated that certain comorbidities are more often linked to a specific phenotype of asthma (267). The most prevalent comorbidity was CRS (29%) followed by obesity (23%) and GERD (18%). OSAS and GERD overlapped with obesity.

Our study is quite unique in the field of asthma as data with sputum RNA-Seq and mRNA cytokine levels are scarce due to the fact that the success rate and quality of sputum samples are sometimes low. Patients are retrospectively studied. Hence, an underestimation of the reported comorbidities is plausible, since only a doctor-diagnosed approach could be used to examine the comorbidities. In addition, only reported environmental exposures reported in the patients file were included. To evaluate the comorbidities and exposures in more detail, a prospective study is required. Another limitation of this study that treatment with glucocorticosteroids was not stopped before the sputum induction as this can influence the sputum transcriptome (39). Due to the fact that the demographics and clinical features are gathered retrospective, no data was collected about severity of asthma and asthma controls scores leaving a gap in the analysis of the bulk RNA-Seq data. Furthermore, we are aware of the fact that the controls seem relatively poorly matched to the patients (age and BMI) and that this might influence the shaping of inflammation due to immune-senescence. However,

this effect was mainly seen in non-atopic athletes above the age of 50 years (282), which includes only 15 patients of our cohort (14%) and therefore considered to have a limited impact.

In conclusion, we found elevated levels of epithelial damage in patients with asthma compared with controls. Moreover, exposure to external irritants impacted sputum transcriptome of asthmatic patients. Smoking resulted in upregulation of CYP1B1 and downregulation of epithelial tight junctions. Equivalently, exposure to cleaning products seem to be associated to the Aryl hydrocarbon pathway.

5. Supplemental material

Study design and subject characteristics

Patients of the outpatient clinic of Pulmonology and Allergology UZ Leuven (Internal Medicine division) who underwent sputum induction for clinical purposes, consented to donate the leftovers of a sputum sample for scientific purposes to the Leuven Allergy and Clinical Immunology Research group (LACI). The samples were stored on RNA-lysis buffer (Qiagen, Westburg) and were collected between October 2010 and March 2019. No treatment stop was needed to enter this study. Asthma diagnosis was based on symptoms, on previous (< 24 months) or current proof of reversibility of $FEV_1 \geq 12\%$ after inhalation of salbutamol according to GINA guidelines and/or a positive histamine provocation test (PC_{20} < 8 mg/ml) and confirmed by a physician based on patient files (24). All healthy subjects were between 18 and 65 years old at the time of the sputum induction and no other pulmonary diseases were diagnosed by a physician. In total 103 sputum samples from asthmatic patients were included in this study together with 34 healthy control samples. By coincidence, 6 patients were already included in the previous study by Seys *et al*. (183), but the new sputum sample used for this study dated from a later time point. The healthy control subject population consists of left-over sputum stored at -80°C in RNA lysis buffer from two previous studies: the study by Seys *et al.* (n = 20) and the study by Decaesteker *et al*. (manuscript in preparation, S61661) (n = 16) and 2 newly recruited samples. However, new cDNA processing and cytokine analysis was performed for all stored mRNA samples.

Retrospective analysis

Spirometry was used to determine lung volumes of all participants and executed according to ERS/ATS guidelines. All lung function parameters such as forced expiratory volume after 1 second (FEV_1), forced vital capacity (FVC), the Tiffeneau index (FEV_1/FVC) and fractional exhaled nitric oxide (FeNO) together with clinical characteristics (age, gender, body mass index (BMI), smoking status, allergic status, treatment and comorbidities) were retrospectively looked up in the patient's files. For the smoking status, a distinction was used between active smoking, ex-smoker or non-smoker. A non-smoker was a person who smoked less than 100 cigarettes in total throughout his life. An active smoker was defined as a person who was still smoking in the last 4 weeks (>100 cigarettes in total) and a former-smoker as a person who did not smoke in the last 4 weeks (>100 cigarettes in total) and had less than 10 pack years.

Allergy to medication was not included in this analysis. The sputum differential cell count was retrospectively looked up in the patients' data files and in a separate database for the healthy controls. Eosinophilic asthma was defined as sputum eosinophils > 3% and sputum neutrophils > 61%, neutrophilic asthma as sputum eosinophils < 3% and sputum neutrophils > 61%, pauci-granulocytic asthma as sputum eosinophils < 3% and sputum neutrophils < 61% and mixed granulocytic asthma as sputum eosinophils > 3 % and sputum neutrophils > 61%.

Bulk RNA sequencing and analysis

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).

Single-end reads of 50 bp length were produced with a minimum of 1M reads per sample. 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 v1.0531. 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 (259). 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 (260). Reported p-values were adjusted for multiple testing with the Benjamini-Hochberg procedure, which controls false discovery rate (FDR). Mean normalized 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 Ontology (GO) analysis was performed with Panther Classification System (283). Gene Set Enrichment Analysis (GSEA) was performed with GSEA software (MSigDbv6, Broad Institute) using the full gene lists with normalized reads.

Table E1: Correlations with lung function parameters.

Correlations with FEV_1%	**Spearman r**	**P-value**
% Neutrophils	-0.1334	0.1881
% Eosinophils	-0.2817	**0.0047**
Uric acid (µM)	-0.0496	0.6549
SP-D (pg/mL)	-0.2326	**0.0292**
HMGB-1 (pg/mL)	-0.2609	**0.0152**
Calprotectin (pg/mL)	-0.2298	**0.0366**
Substance P (pg/mL)	0.0723	0.5156
Correlations with TI (%)	**Spearman r**	**P-value**
% Neutrophils	-0.1445	0.1535
% Eosinophils	-0.4030	**<0.0001**
Uric acid (µM)	-0.1508	0.1607
SP-D (pg/mL)	-0.1908	0.0750
HMGB-1 (pg/mL)	-0.1280	0.2403
Calprotectin (pg/mL)	-0.1166	0.2940
Substance P (pg/mL)	0.0666	0.5481

P-values were corrected by Bonferroni multiple testing.

Table E2: Top 30 differentially expressed genes (padj < 0.05, Log2FoldChange < 1 or > 1): Never vs active smoking patients with asthma

Genes UP	Log2FoldChange	**Genes DOWN**	Log2FoldChange
CYP1B1	3.7	SPRR2F	-5.5
SEMA6B	2.2	SPRR2E	-5.2
FGFR1	2.1	SPRR2A	-5.0
HSPA1B	2.0	SPRR1B	-4.9
DNAJB1	1.8	TRIM29	-4.3
HSPA1A	1.8	CYR61	-3.9
ZFAND2A	1.8	SH3BGRL2	-3.9
HSPH1	1.8	CRCT1	-3.8
BAG3	1.7	S100A14	-3.6
LPAR6	1.7	KRT223P	-3.3
RP11-1008C21.1	1.6	SPINK5	-3.2
SMIM3	1.4	ECM1	-3.2
ABI3	1.4	BAIAP2L1	-3.2
HK2	1.4	ALDH1A3	-3.2
FAM20C	1.3	CLDN4	-3.2
HSPE1	1.3	PDZD2	-3.2
HMOX1	1.3	TMPRSS11B	-3.2
CACYBP	1.3	C6orf132	-3.2
ATF3	1.1	PCDH1	-3.2
TIPARP	1.0	VGLL3	-3.2
SLC1A4	1.0	PAX5	-3.1
GSN	1.0	LIPH	-3.0
		KRT19	-2.9
		EFNA5	-2.8
		MID1	-2.8
		FOXP2	-2.7
		CGNL1	-2.7
		S100A16	-2.7
		HBB	-2.7
		TJP1	-1.8

Table E3: Top 30 differentially expressed genes (padj < 0.05, Log2FoldChange < 1 or > 1): Former vs active smoking patients with asthma

Genes UP	Log2FoldChange	**Genes DOWN**	Log2FoldChange
CYP1B1	3,4	VGLL3	-3,1
SERPINB2	3,4	TCEA3	-2,1
P2RY6	2,5	ITIH5	-2,1
SEMA6B	1,9	LAMB1	-2,0
HMOX1	1,5	PLTP	-2,0
TIPARP	1,1	ARHGAP24	-1,7
		ATP6AP1L	-1,7
		KLHDC8B	-1,3
		NFIA	-1,3

Table E4: Top 30 differentially expressed genes (padj < 0.05, Log2FoldChange < 1 or > 1): Never versus former smoking patients with asthma

Genes UP	Log2FoldChange	**Genes DOWN**	Log2FoldChange
NA	NA	SPRR2E	-4.9
		SPRR2A	-4.4
		SPRR1B	-4.4
		SPRR2D	-4.4
		SPRR2F	-4.2
		KRT78	-4.2
		TMPRSS11A	-4.1
		CRNN	-4.0
		C2orf54	-4.0
		CNFN	-3.9
		PAX9	-3.9
		CYSRT1	-3.8
		TMPRSS11E	-3.7
		S100A14	-3.7
		TMPRSS11B	-3.5
		SPRR3	-3.5
		MAL	-3.4
		KRT4	-3.4
		PRSS22	-3.3
		PADI1	-3.3
		CHAC1	-3.3
		LYNX1	-3.3
		SCEL	-3.3
		MUC21	-3.2
		TMPRSS11D	-3.2
		NCCRP1	-3.2
		CRCT1	-3.2
		RHCG	-3.2
		KRT13	-3.2
		PRSS27	-3.2

Table E5: Differentially expressed genes (padj < 0.05, Log2FoldChange < 1 or > 1): Non-exposure vs cleaning product exposure in patients with asthma

Genes UP	Log2FoldChange	**Genes DOWN**	Log2FoldChange
AHRR	1.0	DDX3Y	-4.2
		FCGBP	-4.5
		SPRR1B	-4.6
		SPRR2A	-4.5
		FDCSP	-4.4

Chapter 8

Pilot study: Assessment of MRGPRX2 on mast cells in sputum of allergic and non-allergic asthma patients

Janne Goossens, Anne-Charlotte Jonckheere, Ellen Dilissen, Jonathan Cremer, Lieven Dupont, Dominique Bullens

1. Introduction

Asthma is a chronic heterogenous disease characterized by reversible airway obstruction and airway inflammation, resulting in shortness of breath, wheezing and chest tightness (284). Mast cells are tissue resident immune cells with a variety of functions that can respond to external triggers, particularly at interfaces interacting with the external environment such as the airways (38). They play a role in both the innate and the adaptive immunity. As a consequence, mast cells may drive different features of asthma according to their response to the immediate environment, inducing particular pathways (40). Since the first description by Paul Ehrlich in 1878, mast cells have been mainly studied in the pathogenesis of allergic diseases such as asthma (285). However, less is known about the role of the mast cell in non-allergic asthma. Recent research revealed IgE-independent pathways, which may be mediated via the novel Mas related G protein coupled receptor (MRGPRX2). MRGPRX2 is predominantly expressed in mast cells and is activated via a broad range of cationic ligands including drugs, host defence proteins, venoms and neuropeptides such as substance P and hemokenin-1 (38). It was already demonstrated that mast cells express MRGPRX2 in sections of lung autopsies and that asthma severity is associated with increased expression of MRGPRX2 in mast cells (50, 286).

A shift in mast cells from the submucosa to the airway epithelium was identified in patients with asthma, driven by EIB positive asthmatics, associated with the presence of airway hyperreactivity (70). The presence of increased mast cell numbers in induced sputum samples, which is an important non-invasive airway sampling method, was first described by Pin I *et al.* via metachromatic staining (287). In addition, molecular phenotyping based on expression of mast cell markers: tryptase (TPSAB1), chymase (CMA1) and carboxypeptidase A3 (CPA3) has been used to identify mast cells in asthma patients and healthy controls (288). With the availability of transcriptomic data, typical mast cell activator signatures are described to be highly expressed in sputum of severe asthma patients (289). However, the direct measurement of mast cells in sputum is challenging due to relatively low abundance in lumen. Consequently, the knowledge of the role of mast cells, especially in non-allergic asthma and regarding the MRGPRX2 receptor is limited.

The aim of this study was to identify and characterize mast cells and its MRGPRX2 receptor expression by flow-cytometry in induced sputum samples of both allergic and non-allergic

asthma patients compared with healthy controls. Secondly, we studied the correlations between mast cells, lung function data, asthma control and mast cell mediators in those patients.

2. Material and methods

Participants

Healthy controls (n=5), allergic asthma patients (n=5) and non-allergic asthma patients (n=5) were included, after signing the informed consent. All study subjects met the following general criteria: between 18 and 60 years old, non-smoking or ex-smoker for at least 12 months and with less than 10 pack years, no pregnancy and no major cardiovascular diseases. Additionally, exclusion criteria for healthy controls included the presence of other pulmonary diseases and presence of an allergic disease. Patients with asthma were screened during their yearly control visit at UZ Leuven. Specific inclusion criteria for asthma patients were: physician-diagnosed asthma since > 6 months, no other concurrent pulmonary diseases and no treatment with oral corticosteroids or biologicals. Maintenance medication was allowed to be continued before the study visit.

Study design

To characterize mast cells in the airways of patients with asthma and healthy controls, a clinical study was performed at the department of Pneumology, UZ Leuven. The study consists out of 1 visit during which informed consent was signed, questionnaires were filled in, spirometry with reversibility test and sputum induction were performed. The study was approved by the Ethics Committee Research UZ/KU Leuven (S52549).

Spirometry

Spirometric measurements were performed according to ERS/ATS guidelines (207). Predicted values were calculated based on the GLI reference values (208). Spirometry was performed before and 15' after inhalation of salbutamol (400 µg) to assess bronchial reversibility.

Sputum induction

Sputum was induced by nebulizing NaCl in increasing concentrations (3 %, 4 % and 5 %) each for 7 minutes. Before each dose increment, spirometry was conducted, and the process was terminated if the FEV_1 decreased by more than 10%. Using the selected plug technique described by Pizzichini *et al.,* sputum cells were isolated (269). To reduce squamous cell contamination, plugs that appeared to be free of salivary contamination were selected and

were treated with dithiothreitol (DTT) to homogenize and obtain a single cell suspension. After cell isolation, living cell counts were determined using a Bürker hemocytometer with trypan blue staining. Sputum cells were used for RNA isolation and flow cytrometric analysis (see below) and sputum supernatant was stored for mediator analysis (see below). Cytospins with 12500 and 25000 sputum cells, respectively, were prepared for differential cell count (Shandon Centrifuge) and stained (Diff-Quick stain, Thermo Fisher, Massachusettes, USA). A total of 250 inflammatory cells were counted. Three asthmatic patients and one control subject failed to produce a good quality of sputum and were therefore excluded. The groups were completed so that each group consists of 5 participants.

Questionnaires

A questionnaire regarding medication use, allergy, smoking state, recent infections and the Asthma Control Questionnaire (ACQ) (table E1) were filled in (211).

Sample analysis

Sputum cells

The obtained sputum cells were divided into cells for RNA isolation (minimum 250 000 cells) and for flow cytometric analysis. 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 of ckit, CMA1, TPSAB1 and MRGPRX2 were measured using real time qPCR (99, 182, 234–236). Data was normalized to the geometric mean of the reference genes PPIA and RPL13A, determined with RefFinder (256). Newly developed primers and probes are listed in supplementary table E2. cDNA plasmid standards were used to quantify the amount of target gene in unknown samples (257).

Sputum SN

Collected sputum supernatant (SN) was stored at -80°C up to analysis. Accidentally, the supernatant of one asthmatic patient was not stored (MCP6). Levels of β-hexosaminidase were measured in the supernatant. Prostaglandin D2 (PGD2) and neurokinin-A (NKA) were measured in sputum supernatant via ELISA (Abbexa, Cambridge, United Kingdom).

Flow cytometry

Flow cytometry was performed on remaining sputum cells. To determine viability, they were incubated with Fixable Viability Dye eFluor™ 780 (Thermo Fisher Scientific, Walthma, MA, USA). In addition, they were incubated with PE-Cy7 anti-human HLA-DR, PE-Cy5 anti-human CD14, AF700 anti-human CD16, BV711 anti-human CD117, V500 anti-human CD45, PerCP-Cy5.5 anti-human FcεRIα, APC anti-human MRGPRX2 and PE anti-human CD63 together with inactivated plasma to block unspecific binding and with Brilliant stain buffer (BD eBiosciences, New Jersey, USA) for optimal staining conditions for the brilliant violet fluorochromes. The antibodies CD45, CD14 and HLA-DR were purchased from Thermo Fisher Scientific and the others were purchased from Biolegend (San Diego, California). Data was acquired with LSR Fortessa SORP flow cytometer running DIVA software (BD biosciences, Erembodegem, Belgium) and analysed with FlowJo 10.7.1 installation.

Statistical analysis

Statistical analysis was performed using Graphpad prism v.9.1.0 (California, USA). Normal distribution was studied with Shapiro-Wilk test. To compare two groups, Mann-Whitney test was used for non-parametric data and unpaired t-test for parametric data. Kruskal-Wallis test was used to compare three or more groups with non-parametric data. Spearman r correlation was used to assess correlation for non-parametric data. A result was considered to be significant when $p < 0.05$.

3. Results

Subject characteristics

In total 10 asthmatic patients, of which 5 allergic and 5 non-allergic respectively, and 5 healthy controls were included. Subject characteristics are described in table 1. All participants were non-smokers. Allergic asthmatic patients were all multi-sensitised to dog (n = 3), cat (n = 3), house dust mite (n = 3), grass pollen (n = 3) and moisture (n = 1), respectively. As anticipated, asthmatic patients had significant lower FEV_1% predicted, FVC% predicted and Tiffeneau index (TI, FEV_1/FVC, %) compared to healthy controls. All asthmatic patients were described on ICS, however 2 patients reported no use (MCP7 and MCP10). ACQ scores ranging from 1 to 15, did not significantly differ between allergic and non-allergic asthma patients. Sputum differential cell counts were not significantly different in asthmatic patients compared with controls (table 1).

Table 1: Subject characteristics.

	Controls (n=5)	Asthma patients (n=10)	P-value
Gender (M/F)	2/3	6/4	0.6084
Age (years)	42.0 ± 12.9	43.6 ± 13.5	0.8259
BMI	24.6 ± 4.3	27.7 ± 4.6	0.2299
Atopic (n)	0	5	0.1009
ACQ	0	1.2 ± 0.9	0.0010
ICS use	/	8	
FEV_1%	119.2 ± 14.3	88.0 ± 14.6	0.0021
FVC%	118.4 ± 4.7	99.7 ± 15.0	0.0191
FEV_1/FVC (%)	81.1 ± 1.0	71.0 ± 7.6	0.0390
Total number of sputum cells (x10^6)	1 (0.7-3.7)	1.7 (0.8-6.1)	0.3710
Sputum macrophages (%)	75.2 ± 24.4	57.9 ± 26.9	0.2487
Sputum lymphocytes (%)	0.3 ± 0.4	1.5 ± 2.3	0.2557
Sputum neutrophils (%)	24.6 ± 24.5	37.5 ± 22.7	0.3311
Sputum eosinophils (%)	0	2.0 ± 4.7	0.3609

Data is represented as mean with standard deviation for parametric data and median with interquartile range for non-parametric data. P-value was determined wit unpaired t-test for parametric data, Mann-Whitney test for non-parametric data and chi-square test for categorical data.

Sputum mast cells are elevated in allergic asthma

Mast cells were identified as $CD14^{low}$, $CD16^{low}$, HLA-DR^{low}, FcεRIα$^+$ and $CD117^+$ cells (figure 1). We found a significantly higher percentage of mast cells in sputum of patients with asthma, specifically in allergic asthma, compared with healthy controls (figure 2A, E1). In addition, specific mast cell markers were assessed via qPCR (figure 2B). cKit mRNA expression was only detected in one patient with asthma (MCP2), who was indeed a non-allergic asthma patient with a high percentage of $CD117^+$ of $CD45^+$ cells (2.1%). Mast cells where further characterized based on their MRGPRX2 expression (figure 2B). MRGPRX2 protein expression and mRNA levels did however not correlate. In contrast, a strong significant positive correlation between chymase (CMA1) and MRGPRX2 mRNA levels was observed (Spearman r=0.9879, p<0.0001). CMA1 and MRGPRX2 both furthermore correlated significantly with TPSAB1 (tryptase) (CMA1 and TPSAB1: Spearman r=0.838; TPSAB1 and MRGPRX2: Spearman r=0.815).

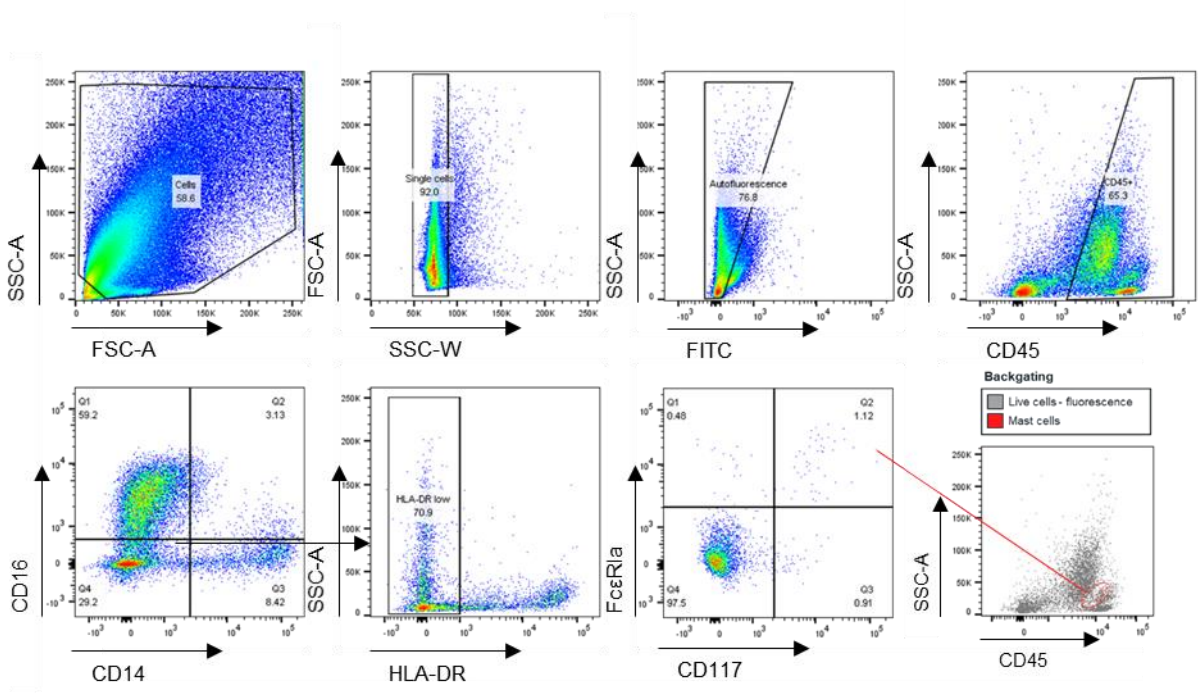

Figure 1: Gating strategy of mast cells in sputum. Mast cells were gated as live, CD45+, CD14low, CD16low, HLA-DRlow, CD117+ and FcεRIα+ cells. The FITC channel was used to detect and eliminate autofluorescence. Backgating of the mast cell population is represented.

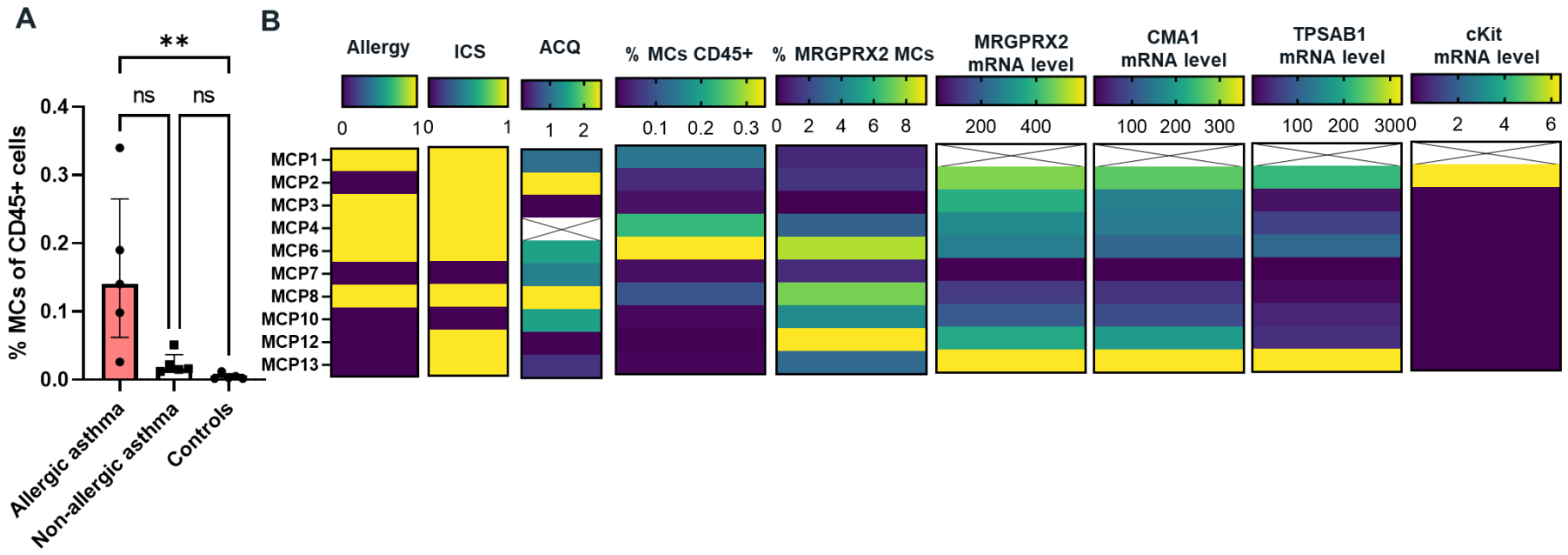

Figure 2: Characterisation of mast cells in sputum. (A) Mast cells expressed as % of live CD45+ sputum cells in allergic asthma patients (n=5), non-allergic asthma patients (n=5) and controls (n=5). **(B)** Heatmap of allergy, inhalation corticosteroid (ICS) use, Asthma control questionnaire (ACQ) score with ACQ score <0.75 for controlled asthma and >1.5 for poorly controlled asthma, % of mast cells of CD45+ cells and mRNA level of CMA1, TPSAB1, MRGPRX2 and cKit in each asthmatic patient. A white box was used to indicate 'not available'. Kruskal-Wallis test was used to determine P-value. ** p <0.01

Mast cell activation markers

To assess the activation state of identified mast cells, PGD2 and β-hexosaminidase were measured in the collected sputum supernatant. Although no significantly elevated numbers of mast cells were observed in non-allergic asthma, a trend towards higher activation levels was observed in patients with non-allergic asthma (figure 3A). Both markers of activation, β-hexosaminidase and PGD2 correlated significantly with each other (Spearman r=0.7500, p=0.0255, Figure 3B). As suggested by recent evidence, neuropeptides may play an important role in mast cell activation and the neuro-immune interaction described in asthma. We observed a trend towards higher NKA levels in allergic asthma patients (figure 3C). Moreover, NKA levels but no other activation levels correlated significantly with the percentage of mast cells defined as % of $CD45^+$ population (figure 3D, table E4)

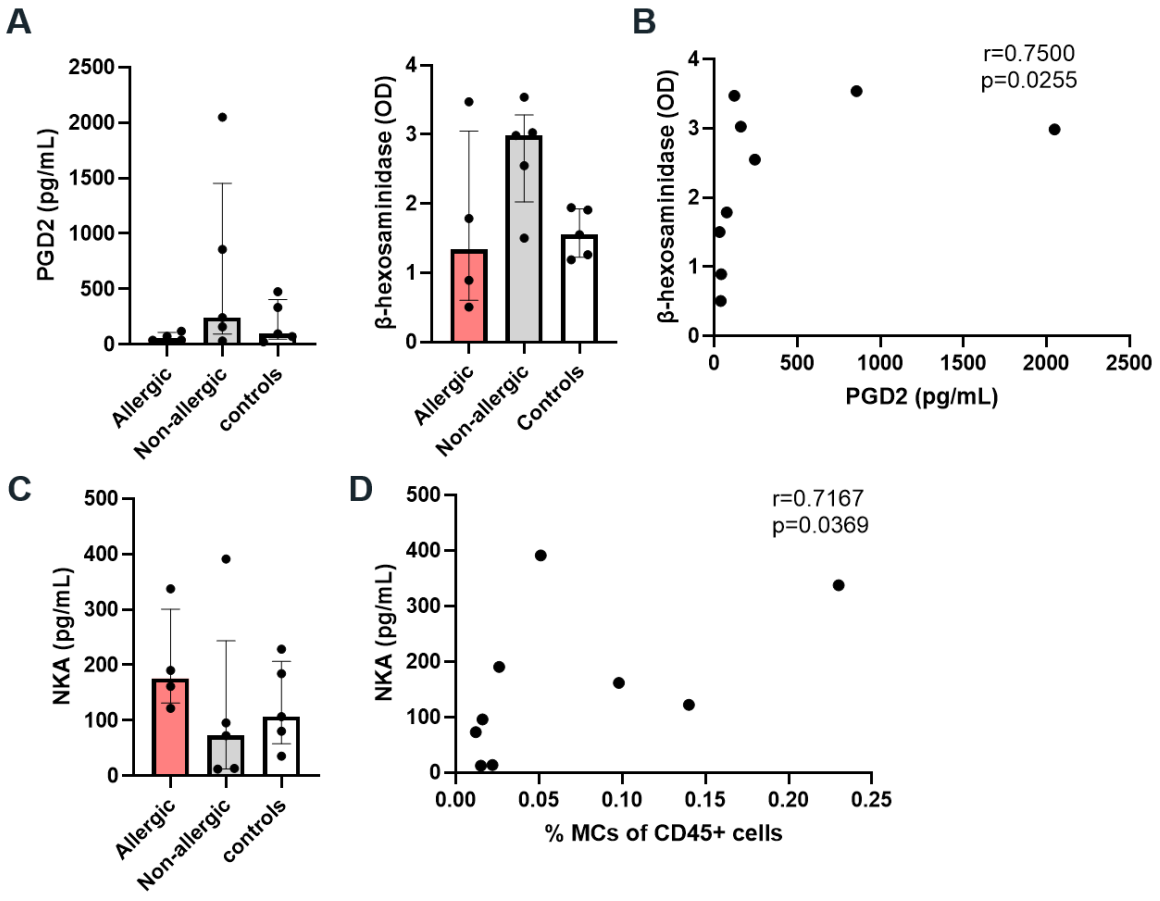

Figure 3: Mast cell associated mediators measured in sputum supernatant. (A) Mast cell activation mediators prostaglandin D2 (PGD2) (pg/mL) and β-hexosaminidase (OD) in sputum supernatant of allergic asthmatic patients (n=4), non-allergic asthmatic patients (n=5) and controls (n=5). **(B)** Correlation between mast activation mediators PGD2 (pg/mL) and β-hexosaminidase (OD) (n=9). **(C)** Neurokinin A (NKA) (pg/mL) in sputum supernatant of allergic asthmatic patients (n=4), non-allergic asthmatic patients (n=5) and controls (n=5). **(D)** Correlation between % mast cell (MC) numbers of CD45+ live cells and NKA levels. Kruskal-Wallis test was used to determine P-value, Spearman correlation.

Clinical associations of sputum mast cells

We examined the clinical characteristics of asthmatic patients in association with the number and activation state of mast cells. The percentage of sputum mast cells correlated negatively and significantly with the number of sputum neutrophils, indicating that we observed more mast cells in patients with low sputum neutrophil counts (figure 4A). Although activated, we did not observe a positive correlation with the number of sputum eosinophils (figure 4A), probably due to the low number of eosinophils counted in our cytospin slides. We observed that percentage of sputum mast cells correlated negatively and significantly with FEV_1% predicted (figure 4B, table E5). Moreover, a significant negative correlation was observed between NKA levels and TI (%) but not with FEV_1% (figure 4B, table E5).

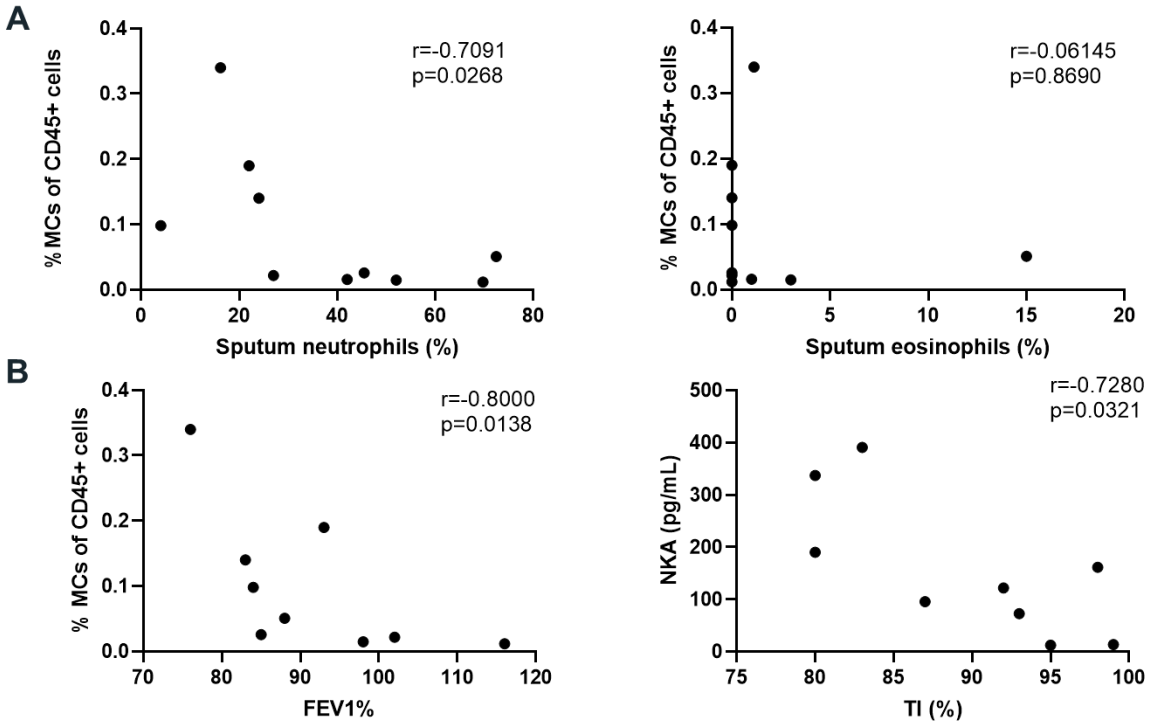

Figure 4*:* **Correlation between mast cells and clinical characteristics. (A**) Correlation between percentage mast cells (MCs)of CD45+ live sputum cells and sputum counted neutrophils and eosinophils, respectively. **(B)** Correlation between percentage of mast cells of CD45+ live cells and lung function parameter FEV_1%. Correlation between neurokinin A (NKA) and lung function parameter Tiffeneau index (TI). Spearman correlations.

4. Discussion

In this pilot study, we were able to characterize mast cells in sputum samples of asthmatic patients and healthy controls by flow cytometry. Our observed percentages of mast cells were in line with Fricker *et al.* (290), which is by our knowledge the only publication focusing on sputum mast cells by flow cytometry. As all mast cell percentages were below 0.5%, this highlights that mast cells are a rare cell population in sputum samples. Other findings on mast cells in sputum in literature are based on transcriptomic profiles (66, 288, 291). A recent study by flow cytometry demonstrated that a significant portion of airway progenitor cells in induced sputum samples are mast cell precursors in patients with allergic asthma (292). This is in line with our observation of significantly elevated mast cell percentage in allergic asthma patients compared to healthy controls.

The MRGPRX2 receptor was not specifically associated to neither allergic nor non-allergic asthma, indicating that in both phenotypes, there might be a role for IgE-independent mast cell activation via MRGPRX2. In addition, MRGPRX2 mRNA levels strongly correlated with CMA1 mRNA levels, which is in accordance with the finding that MRGPRX2 can be used as signature marker of the mast cell subtype MC_{TC} (47). Recently, it was demonstrated that the infiltration of particular chymase-high mast cells was associated with the degree of airway hyperresponsiveness in corticosteroid-free patients (293). Different distribution of mast cells was observed in patients with high FeNO levels, in which MC_{TC} were elevated in airway epithelium, and low FeNO levels in which MC_{TC} were elevated in airway smooth muscle (293). There was no significant correlation between mast cells markers studied via flow cytometry and qPCR, indicating that there is a discrepancy between gene expression and surface expressing markers, at least in this small population. Regarding the mast cell activation markers, we found a tendency towards an elevated level of mast cell activation in non-allergic asthma. Although the low numbers of mast cell in non-allergic asthma, they might be in an active state contributing to airway inflammatory response. It is important to note that all allergic asthma patients and 3/5 of non-allergic asthma patients were using ICS, which can influence mast cell activation. In particular, ICS are known to reduce recruitment and activation of Th2 cells, eosinophils and mast cells (294).

NKA is a neuropeptide produced by the same gene as substance P, but alternatively spliced. Those neuropeptides are respectively preferential agonists for receptors neurokinin-1 (NK1)

and NK2, and are present on airway mast cells (295). We found a significant negative correlation between NKA levels and TI. Similarly, a negative correlation was observed between substance P and FEV_1/FVC in induced sputum samples of asthmatic patients (296). These data suggest that neurogenic inflammation may be involved in the inflammatory reaction and subsequent airway narrowing. This hypothesis is further evidenced by the finding that the percentage of sputum mast cells correlated with NKA levels, indicating that we observed higher mast cell numbers in patients with higher neuropeptide levels. Also the MRGPRX2 receptor on mast cells is described to have neuropeptides as ligand such as substance P and hemokinin-1. Recent evidence suggests that substance P activates mast cells primarily through the MRGPRX2 receptor (297). These finding shows how mast cells and the newly identified MRGPRX2 receptor may participate in neurogenic inflammation in asthma. Less research is known on NKA and the MRGPRX2 receptor. As NKA and substance P are highly homogenous and the NK receptors have affinity for both substance P and NKA (298), it cannot be excluded that the MRGPRX2 may also be activated via NKA.

The association of mast cells with clinical characteristics of asthma suggest that they might be useful in the clinical management of asthma. However, we could not find a correlation between mast cell activation markers and lung function. A possible explanation for this, is the low sample size included. Although β-hexosaminidase is considered to be mast cell specific, PGD2 can also be produced by other immune cells such as Th2 cells and dendritic cells (299). Of note, protein levels may be underestimated due to the effect of DTT on the ELISA as it is known that DTT can reduce detectable protein concentrations in sputum supernatants (300). This study has some more limitations. First of all, these are preliminary data of a small cohort. Further validation in an independent and larger cohort is necessary. The present study was cross-sectional, while a longitudinal study following mast cells over time is recommended. Furthermore, not every patient used the same medications which can bias the results. However, none of them were on OCS or used biologicals.

In conclusion, we characterized mast cells expressing MRGPRX2 using flow cytometry. Mast cells were elevated in allergic asthma compared with controls. While for non-allergic asthma patients also mast cell activation was observed. Sputum mast cells correlated with NKA and associated with lower lung function. This preliminary data demonstrates the role of mast cell should be further studied in relation to neuro-immune interaction in both allergic and non-allergic asthma.

5. Supplementary material

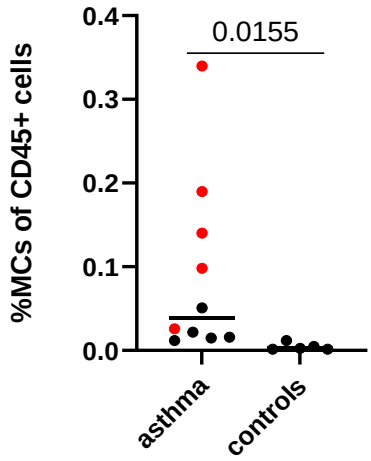

Figure E1*:* Mast cells in asthma patients and controls. Red dots represent allergic asthma patients. Mann-Whitney test was used to determine P-value.

Table E1: Asthma Control Questionnaire (ACQ) (Dutch validated translation)

Hoe vaak bent u per nacht gemiddeld wakker geworden door uw astma in **de afgelopen week**?	0 Nooit 1 Bijna nooit 2 Een paar keer 3 Verscheidene keren 4 Vaak 5 Heel vaak 6 Kon niet slapen vanwege astma
Hoe ernstig waren uw astmaklachten bij het 's morgens wakker worden gemiddeld in **de afgelopen week**?	0 Geen klachten 1 Heel lichte klachten 2 Lichte klachten 3 Matige klachten 4 Vrij ernstige klachten 5 Ernstige klachten 6 heel ernstige klachten
In welke mate werd u over het algemeen in **de afgelopen week** door uw astma beperkt bij uw activiteiten?	0 Helemaal niet beperkt 1 Nauwelijks beperkt 2 Een beetje beperkt 3 Tamelijk beperkt 4 Erg beperkt 5 Heel erg beperkt 6 Volledig beperkt
In welke mate heeft u zich over het algemeen kortademig gevoeld in **de afgelopen week** ten gevolge van uw astma?	0 Helemaal niet 1 Nauwelijks 2 Een beetje 3 Middelmatig 4 Vrij ernstig 5 Ernstig 6 Heel ernstig
Hoe vaak had u in **de afgelopen week** over het algemeen een piepende ademhaling?	0 Nooit 1 Zelden 2 Af en toe 3 Geregeld 4 Vaak 5 Meestal 6 Altijd
Hoe veel puffs/inhalaties van een kortwerkend luchtwegverwijdend middel (bijv. ventolin, salbutamol of airomir) heeft u op de meeste dagen genomen in **de afgelopen week**?	0 Geen 1 1-2 puffs/inhalaties 2 3-4 puffs/inhalaties 3 5-8 puffs/inhalaties 4 9-12 puffs/inhalaties 5 13-16 puffs/inhalaties 6 meer dan 16 puffs/inhalaties
Tel uw punten op om uw totaalscore te berekenen.	**TOTAAL:**

Table E2: Primer and probe sequences for qPCR

Gene		Sequence
CMA1	FW	tct gca ttt aag gga gac tct gg
	RV	att cgg gtg aag aca gca gg
	TP	ccc agg gca tcg tat cct atg gac g
TPSAB1	FW	gtg gac aat gat gag cgc ct
	RV	gac gtc gtc tcc cgt gta gg
	TP	cca ccg cca ttt cct ctg aag ca
MRGPRX2	FW	agg gtg tta agg ggc acc ag
	RV	ctc ctt gcc aca aag cag aag
	TP	agt aca aca gtg aat gga aat gac caa gcc c

Table E3: Correlations with mast cell mediators

Correlations with %MCs of CD45+ cells	Spearman r	P-value
NKA (pg/mL)	0.7167	0.0369
PGD2 (pg/mL)	-0.5167	0.1618
B-hexosaminidase (OD)	-0.5500	0.1328

Table E4: Correlations with lung function parameters

Correlations with FEV_1%	Spearman r	P-value
%MCs of CD45+ cells	-0.8000	0.0138
NKA (pg/mL)	-0.3667	0.4068
PGD2 (pg/mL)	0.8333	0.4555
B-hexosaminidase (OD)	0.450	0.4068
Correlations with TI (%)	**Spearman r**	**P-value**
%MCs of CD45+ cells	-0.3212	0.3679
NKA (pg/mL)	-0.7280	0.0321
PGD2 (pg/mL)	0.5021	0.1711
B-hexosaminidase (OD)	0.1172	0.7659

P-values were corrected by Bonferroni multiple testing.

Chapter 9

Optimization of human mast cell differentiation to study MRGPRX2-induced activation *in vitro*

Manuscript in preparation

Janne Goossens*, Toon Ieven*, Ellen Dilissen, Jonathan Cremer, Anne-Charlotte Jonckheere, Willem Roosens, Lieven Dupont, Dominique Bullens

*Contributed equally

1. Introduction

Mast cells are rare tissue resident cells widely described for their role in allergic disease (40). Human mast cells are derived from $CD34^+$ progenitor cells in the bone marrow. Next, these progenitors will enter the bloodstream in undifferentiated state and migrate to target tissues (40). Here, differentiation of specific mast cell committed progenitors, described as $CD34^+$ $CD117^+$ $FcεRI^+$ cells, into tissue-specific mature mast cell subpopulations will take place (301). Typically, mast cells are subdivided based on serine proteases into MC_T, containing tryptase and MC_{TC} containing tryptase and chymase (38).

The most studied mast cell activation pathway is the IgE-mediated pathway, in which activation is caused by allergen cross-linking of IgE bound to FcεRI receptors, leading to the release of different mediators (302). However, mast cell related inflammatory conditions might also include IgE-independent activation e.g. via newly described Mas-related G protein–coupled receptor X2 (MRGPRX2) (42). Nonetheless, the MRGPRX2 mediated mast cell activation is still incompletely understood and difficult to explore. Much of our current knowledge is based on mice models and cell lines (47). To gain more insights int the role of mast cells in non-IgE mediated conditions and their interaction with other immune cells, human *in vitro* differentiated mast cells can help. Published differentiation protocols can take up several weeks and can generate different phenotypes as mast cells are sensitive to their environment (82, 84, 85). Therefore, there is a need for shorter and efficient differentiation protocols, resembling human mast cells. Recently, a promising shorter protocol (3 weeks) was published by Cop *et al.* (87) only using SCF and IL-3 as supplemented cytokines in culture medium. Lappalainen *et al.* demonstrated that IL-6 is helpful too for developing mast cells to differentiate into a more mature phenotype, which can be helpful to shorten the differentiation protocol. In addition, adding FBS to later weeks of culture is described to increase viability of obtained mast cells (86).

The aim of this study was to optimize mast cell differentiation of $CD34^+$ progenitors to obtain functionally MRGPRX2 expressing mast cells. Moreover, differences in mast cell yield between fresh peripheral blood samples and buffy coat as starting material were also studied. Specifically, the duration of the protocol and the role of IL-6 and FBS were investigated. Functionality was assessed via assays containing both IgE- and MRGPRX2-mediated mast cell

activation. Cultured mast cells could be useful to develop diagnostic mast cell activation tests or their specific activation and/or inhibition by specific compounds can be tested.

2. Methods

In vitro culture

Human mast cells were cultured according to Cop *et al.* (87) and Lappalainen *et al.* (83) with modifications (figure 1). Briefly, human mast cells were cultured from progenitors of both buffy coat (BC, ± 50 mL, n=8, supplied by Red Cross Donor Centrum, Belgium) and fresh peripheral blood (PB) samples (80 mL, n=18) of healthy donors. All donors gave their written informed consent as approved by the Ethics Committee of UZ/KU Leuven (S62076). First, peripheral blood mononuclear cells (PBMCs) were isolated from blood samples using density gradient centrifugation with Lymphoprep (Stemcell Technologies, Vancouver, Canada). Next, the Easysep $CD34^+$ Selection Kit (Stemcell Technologies, Vancouver, Canada) was used to select $CD34^+$ cells in accordance with the manufacturer's instructions. Isolated $CD34^+$ progenitor cells were cultured in a serum-free methylcellulose-based medium (MethoCult human SF H4236) (Stemcell Technologies, Vancouver, Canada) supplemented with low density lipoprotein (LDL, 10 µg/mL) (Stemcell Technologies, Vancouver, Canada), β-mercaptoethanol (55 µmol/L) (Sigma Aldrich, Missouri, USA), penicillin-streptomycin (100 U/mL) (Life Technologies, Waltham, USA) and stem cell factor (SCF, 100 ng/mL) (Miltenyi Biotec, Bergisch Gladbach, Germany). In addition, the medium was supplemented with IL-3 (100 ng/mL) and eventually IL-6 (100 ng/mL) (Peprotech, New Jersey, USA) and incubated for 3 or 4 weeks at 37°C and 5% CO_2 with high seeding density (1 - 2 x 10^5 cells/mL) in 6 well plate (for BC) and low seeding density (1 -2 x 10^4) in inner wells of 24 well plate with each 1.1 mL/well. At day 3, 7, 10, 14 and 17 cells were nourished by adding 300 µl of Iscove's Modified Dulbecco's Medium (IMDM) (Life Technologies, Waltham, USA) containing penicillin-streptomycin (100 U/mL), 1% Insulin-Transferrin-Selenium (Life Technologies, Waltham, USA), 0.1% Bovine Serum Albumin (Sigma Aldrich, Missouri, USA), SCF (20 ng/mL) and eventually IL-6 (20 ng/mL) on the serum-free methylcellulose-based medium. After 3 weeks, cells were retrieved and cultured in IMDM medium containing SCF (20 ng/mL) with or without FBS (5%) at a density of 0.5 x 10^6 cells/mL for a one week during resting phase. When adjusting the duration of the protocol, the period in serum-free methylcellulose based medium with the addition of nourishing medium was adapted. Thus, the last week remains a resting phase.

Depending on the quality and yield of each culture, different numbers of experiments could be accomplished.

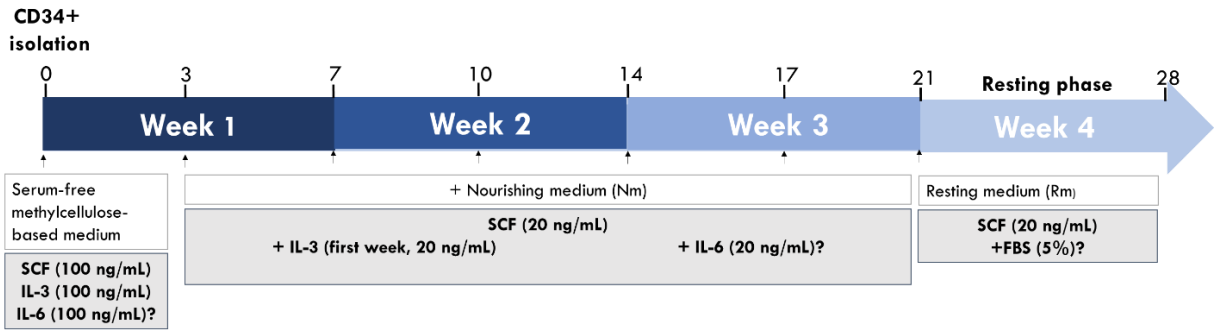

Figure 1: Overview mast cell differentiation protocol for 4 weeks. CSF, Stem cell factor. FBS, fetal bovine serum.

Immunophenotyping

Flow cytometry was performed on differentiated mast cells (during or after resting phase). To determine viability, they were incubated with Fixable Viability Dye eFluorTM 780 (Thermo Fisher Scientific, Walthma, USA). In addition, for extracellular characterisation they were incubated with BV711 anti-human CD117, PerCP-Cy5.5 anti-human FcεRIα, APC anti-human MRGPRX2, BV421 anti-human CD203c and PE anti-human CD63 together with inactivated plasma to block unspecific binding and with Brilliant stain buffer (BD eBiosciences) for optimal staining conditions for the brilliant violet fluorochromes. To characterize mast cell progenitors, FITC anti-human CD34 was added. The antibody CD45 was purchased from Thermo Fisher Scientific (Walthma, USA) and the others were purchased from Biolegend (San Diego, California). For intracellular characterisation, anti-human tryptase primary mouse and anti-human chymase primary rabbit antibodies (Abcam, Cambridge, UK) were used in combination with AF488 donkey anti-mouse and AF594 donkey anti-rabbit secondary antibodies (Invitrogen, Waltham, USA). Data was acquired with LSR Fortessa SORP flow cytometer running DIVA software (BD biosciences, Erembodegem, Belgium) and analysed with FlowJo 10.8 installation. Mast cells were identified as $CD117^+$ $CD203C^+$ cells (figure E1).

Cell staining

Cytospin slides were made (Shandon cytocentrifuge) and stained with Diff-Quick (Thermo Fisher, Waltham, USA) and Toluidine Blue (Sigma Aldrich, Missouri, USA) staining. Specifically, 50.000 cells were loaded and centrifuged for 4 minutes at 800 rpm to immediately perform Diff-Quick staining according to manufacturers' guidelines. For toluidine blue staining, also 50.000 cells were loaded and centrifuged for 10 minutes at 800 rpm. Next, cells were fixed

with Clarke's fixation (75% ethanol and 25% acetic acid) for 10 minutes. After rinsing, cells were stained with acidic toluidine (pH < 1) for 20 minutes at room temperature. Then slides were rinsed and air-dried.

Functional analysis

During and after the resting phase, the function of human differentiated mast cells was evaluated via IgE and non-IgE dependent stimuli, including substance P and compound 48/80 (C48/80). For stimulation with anti-IgE, cells were sensitized overnight with human serum at 37°C and 5% CO_2. Next, cells were washed and resuspended in pre-warmed hepes buffer at a density of 0.5 x 10^6 cells/mL. Subsequently, sensitized cells were incubated with polyclonal goat anti-human anti-IgE (1 µg/mL) (Sigma Aldrich, Missouri, USA) for 1 hour at 37°C in hot water bath. For non-IgE mediated activation, unsensitized mast cells were resuspended in hepes buffer at density of 0.5 x 10^6 cells/mL and incubated with substance P (0.1 mg/mL) (Sigma Aldrich, Missouri, USA) or C48/80 (2.5 µg/mL) (Sigma Aldrich, Missouri, USA) for 20 minutes at 37°C in hot water bath. Optimal concentrations were defined based on dose-response curve. For inhibition experiments, differentiated mast cells were incubated with ketotifen (100 µM) (Sigma Aldrich, Missouri, USA) or cromolyn sodium (1000 µM) (Sigma Aldrich, Missouri, USA) for 15 minutes on 37°C in hot water bath just before addition of the stimuli. Reactions were stopped by placing cells on ice for 5 minutes and centrifugation (5' at 400G). The supernatant was removed and cells were stained by flow cytometry like described above.

Statistical analysis

The entire analysis was carried out with the aid of Prism v.9.1.0. (California, USA). Data were presented as median with IQR. Comparisons between groups were performed with Mann-Whitney test. To assess correlations, Spearman analysis was performed. A result was considered to be statistically significant if $p<0.05$.

3. Results

Progenitor characterisation

First of all, the numbers of mast cell CD34⁺ progenitors starting from fresh PBMC compared to buffy coats, were studied. Mast cell progenitors are the starting cells for the mast cell differentiation protocol. Subsequently, CD34⁺ cells were isolated via immunomagnetic selection (IMS) of PBMCs of both buffy coat (BC, +/- 50 mL) and peripheral blood (PB, 80 mL) (figure 2A). Buffy coats contained significantly higher number of PBMCs and CD34⁺ progenitor cells ($p<0.005$)(figure 2B). However, the proportion of isolated cells after IMS on live PBMCs did not differ amongst the two donor types. A significant negative correlation was found for the proportion of isolated CD34⁺ progenitor cells and age of the donors. We did not find a significant difference for the proportion of isolated cells between male and female donors.

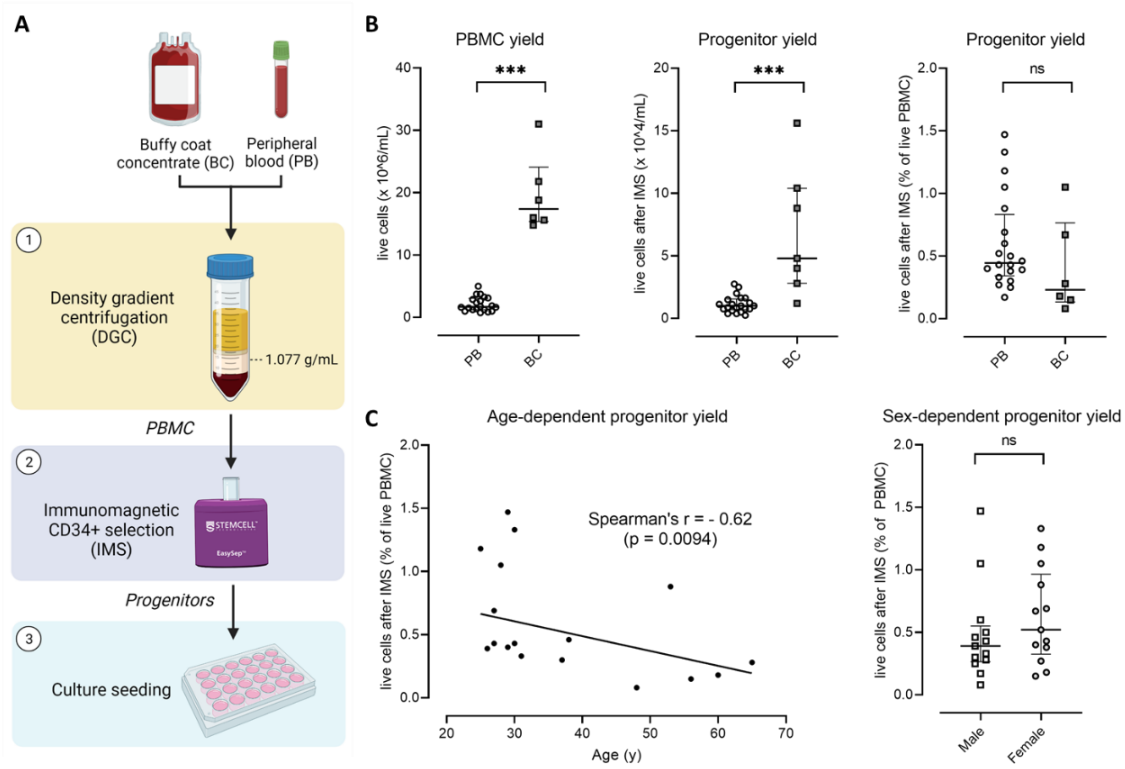

Figure 2: Progenitor yield. (A) Mast cell progenitor selection methodology. **(B)** Manual counts of live cells stained with trypane blue after PBMC isolation trough Lymphoprep density gradient centrifugation and immunomagnetic selection of CD34⁺ progenitor cells. **(C)** Progenitor yield in association with age (y) and gender. Data represented as median with IQR. Horizontal bar above graphs indicates comparison between groups through Mann-Whitney U test.
*** = $p < 0.005$, ns = $p > 0,05$ (not significant). BC, buffy coat; PB, peripheral blood; PBMC, peripheral blood mononuclear cells; y, years.

Next, we studied the isolated fractions of IMS via flow cytometry (figure 3A). Indeed, the negative fraction (supernatant) barely contained CD34⁺ cells. For the positive fraction, which was further used during the protocol, the CD34⁺ yield ranged from 15% up to 50% (figure 3B). The percentage committed mast cell progenitors (CD34⁺, FcεRIα⁺, CD117⁺) amongst live cells varied from 1.5% to 10%.

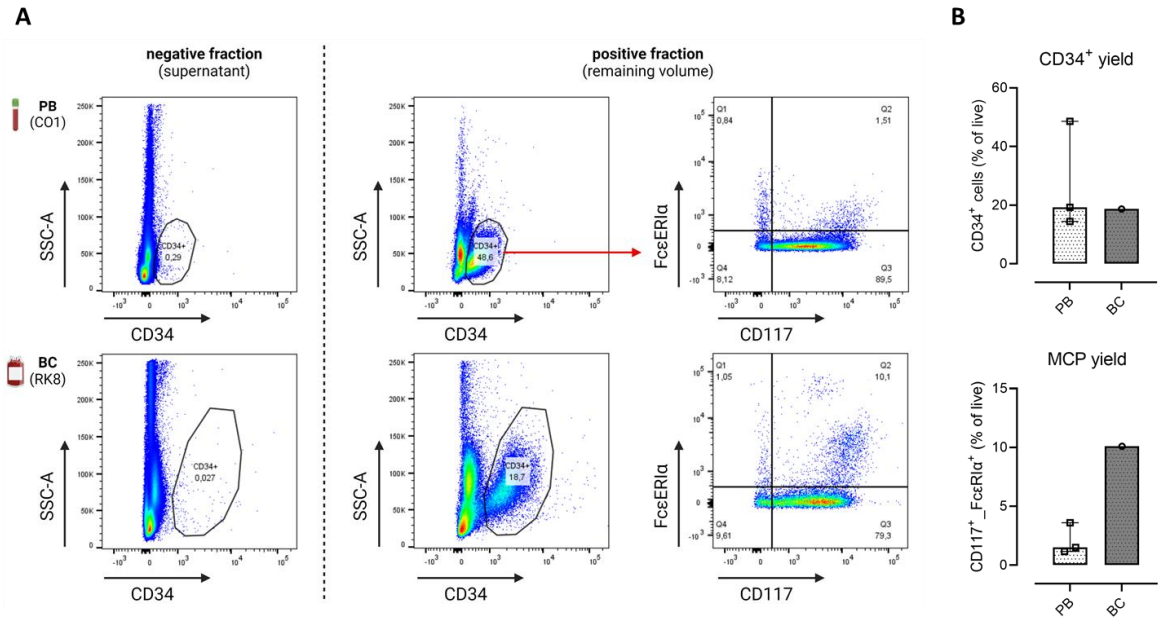

Figure 3: Immunophenotyping of fractions after immunomagnetic CD34⁺ selection. (A) Representative dot plot graph for negative and positive fraction after immunomagnetic selection of CD34+ cells with Stemcell Easysep selection kit of PBMCs of peripheral blood (PB) and buffy coat (BC). In positive fraction, mast cell committed progenitors (MCP) were identified as CD117⁺ and FcεRIα⁺ CD34+ cells. **(B)** Proportions (among live cells) of CD34+ and MCP in PB and BC. Data are represented as median with IQR.

Mast cell differentiation

Next, the protocol for mast cells differentiation was optimized. The duration of the protocol was found to be optimal for 4 weeks, showing significantly higher viability and similar CD117, FcεRIα and MRGPRX2 expression (figure 4A). MRGPRX2 was expressed on 46% of mast cells cultured in the presence of IL-3 (20 ng/mL during week 1) and IL-6 (20 ng/mL week 1-3) (figure 4C). Addition of IL-6 substantially increased MRGPRX2 expression compared to conditions with only IL-3 (30% of mast cells) (figure 4B). Addition of FBS (5%) in week 4, increased MC viability (45% to 62%) but dramatically decreased MRGPRX2 expression (46% to 3%) (figure 4D). We therefore decided to use the protocol with addition of IL-6 (20 ng/mL) in the nourishing medium without FBS in resting medium in further experiments. So CD34⁺ cells were differentiated into viable mature mast cells containing

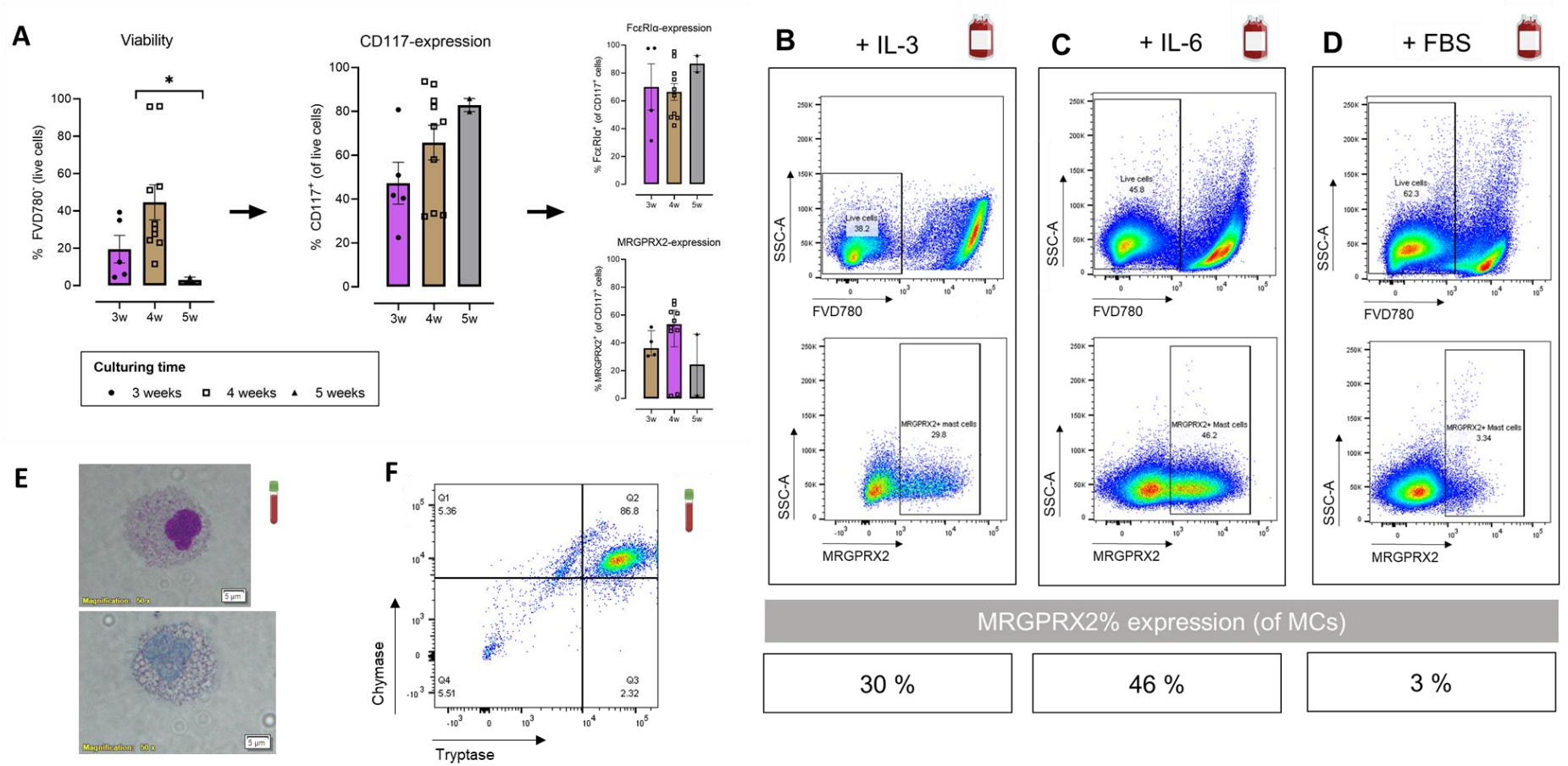

Figure 4: Impact of protocol adaptations. (A) Immunophenotyping of cultured progenitors after 3 (n=5), 4 (n=10) or 5 (n=2) weeks in medium. Results are indicated as median with IQR. Mann-Whitney test; * = $p < 0,05$. Representative plots of immunophenotyping of obtained mast cells after addition of IL-3 (20 ng/mL) in first week of nourishing medium (Nm) **(B)**, after addition of IL-6 (20 ng/mL) in Nm **(C)**, after addition of IL-6 (20 ng/mL) in Nm and FBS (5%) in resting medium (Rm) **(D)**. **(E)** Representative images of obtained mast cells stained by diff-Quick (upper) en Toluidine (lower). **(F)** Representative plot of intracellular staining of obtained mast cells with tryptase and chymase.

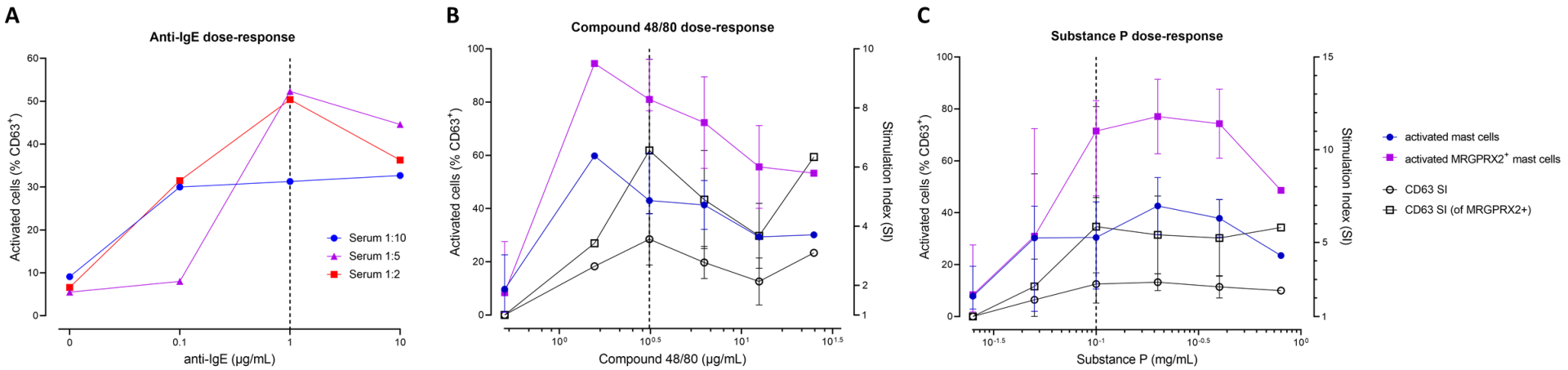

Figure 5: Dose response curves for stimulation experiments. (A) IgE mediated activation of mast cells via anti-IgE. Mast cells were passively sensitized overnight (37°C, 5% CO_2) with human serum at 1:2 dilution, 1:5 dilution and 1:10 dilution (n=1). **(B)** Non-IgE mediated stimulation via compound 48/80 stimulation (n=4). Stimulation Index (SI), which is the ratio of % in stimulated condition of % in unstimulated conditions, is expressed on second x-axis. **(C)** Non-IgE mediated activation of mast cells via substance P (n=5). SI is expressed on second x-axis. Dotted vertical lines are optimal concentrations. Results are indicated as median with IQR.

granules visualised with microscopy (figure 4E) and containing both intracellular tryptase and chymase assessed with flow cytometry (figure 4F).

Functional validation

In order to study whether the obtained cells could be functionally activated, mast cells were stimulated with anti-IgE, representing IgE mediated activation and with substance P and compound 48/80 (C48/80) representing MRGPRX2 mediated activation. For all stimuli, a dose-response curve was obtained, demonstrating functionality of the mast cells. For anti-IgE stimulation, a serum dilution of 1:5 with 1 µg/mL of anti-IgE was found to be optimal (figure 5A). As determined by CD63 membrane expression, the MRGPRX2 specific C48/80 effectively induced degranulation. However, decreased viability was observed with increasing dose (figure E2), suggesting that lower doses should be used. C48/80 stimulation induced degranulation in specifically MRGPRX2 expressing mast cells with optimal concentration of 3.12 µg/mL (figure 5B). Similarly, substance P induced mast cell activation within MRGPRX2 expressing mast cells (figure 5C) with optimal concentration of 0.1 mg/mL. A representative plot of non-IgE mediated activation in MRGPRX2 expressing mast cells is shown in figure E3.

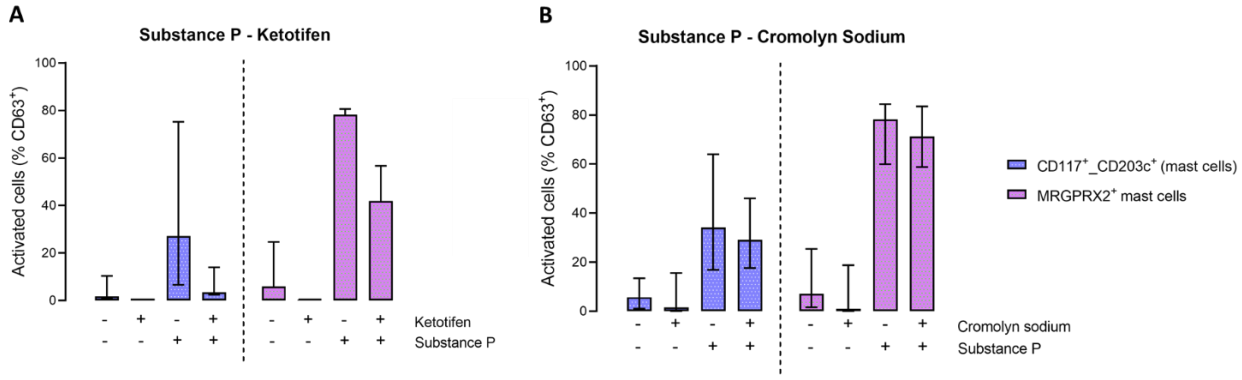

Figure 6: Pre-inhibition of mast cells stimulated with substance P. CD63 expression was shown in full population of mast cells and subset of mast cell expressing MRGPRX2. Mast cells were pre-incubated with Ketotifen (n=4) **(A)** or cromolyn sodium (n=6) **(B)** for 10' at 37°C before stimulation with substance P (0.1 mg/mL). Results are indicated as median with IQR.

The ability of classical mast cell inhibitors on endogenous MRGPRX2-mediated mast cell activation by substance P was assessed. Ketotifen was able to reduce CD63 expression on mast cells (figure 6A), while for cromolyn sodium the inhibition was limited (figure 6B).

4. Discussion

This study demonstrates that adding FBS substantially decreased and IL-6 increased MRGPRX2 expression during human mast cell differentiation *in vitro.* The obtained mast cells were considered to be mature showing granulation and containing both tryptase and chymase. This suggests that obtained mast cells resembles MC_{TC} mast cell subtype, which is described to express MRGPRX2 (38). Furthermore, obtained mast cells were functionally valid allowing for evaluation of both IgE-dependent and IgE-independent (MRGPRX2 mediated) pathways. This enables screening of pharmacological inhibitors.

The differentiation protocol started with either progenitors isolated from buffy coats or peripheral blood. Our culture model allows the generation of viable mast cells starting from a small amount of progenitors. It is advantageous as primary (patient-derived) samples can be used as source for progenitors to obtain mast cells in a relatively short timeframe of 4 weeks, which is cost-effective. Similar relative progenitor yield was obtained in buffy coat and peripheral blood, negatively correlating with donor age. As aging is accompanied by a decline in immune responses, this is not unexpected (303). Other common resources of progenitors are bone marrow or cord blood (304, 305), which are less accessible. Using the commercially available $CD34^+$ immunomagnetic selection kit, $CD34^+$ PBMCs were isolated. However, the mentioned purity of manufacturer (93.5 ± 1.1%) obtained with cord blood was not achieved within our peripheral blood nor buffy coat samples. The committed mast cell progenitor yield, already expressing CD117 and FcεRIα, within these isolated fractions ranged from 1.5 – 10%. However, we believe that also non-committed progenitors may differentiate into mast cells. Keeping differentiating mast cells for a longer period in time (> 4 weeks) in the methylcellulose-based medium resulted in increased cell death. This is contrast with literature, as mast cell differentiation protocols can take up to 8 weeks (82–84), in which a liquid-based medium is applied.

Activation of MRGPRX2, which is expressed on human mast cells, is considered to be important in mast cell degranulation besides cross-linking of IgE/FcεRIα pathway. To obtain highly MRGPRX2 expressing mast cells, IL-6 and FBS were added to the culture medium, promoting mast cell differentiation. Cop *et al.* did not find an effect of IL-6 on non-IgE dependent degranulation (306). However, we found elevated MRGPRX2 expression in mast cells cultured with IL-6. Interestingly, FBS induced reduced MRGPRX2 expression on mast cells.

To our knowledge, this effect was not described before. This finding is relevant for further research focusing on MRGPRX2 mediated pathways *in vitro*. Others investigated the effect of MRGPRX2 inhibition by the use of small interference RNA inducing MRGPRX2 silencing (307). Furthermore, different inhibitors have been developed including direct inhibitor such as QWF and inhibition of MRGPRX2 downstream signalling which might have therapeutic potential (48). We showed that the differentiated human mast cells degranulate in response to neuropeptide substance P. Recent studies indeed have demonstrated that substance P is ligand of the MRGPRX2 receptor on mast cells (286). Substance P is a member of the tachykinin family of neuropeptides and is a well-known ligand for neurokinin 1 and 2 receptor (NK1R, NK2R) with preference for NK1R (308).

As alternative activating agent, C48/80, polycationic molecules, was used to induce MRGPRX2 mediated activation (309). The increased observed cell death, expressed as FVD780 uptake, observed in mast cells stimulated with higher concentration of C48/80 might be explained by toxic effect of those concentrations. Another suggestion might be massive degranulation, enabling FVD780 going intracellular. We did not study whether mast cells could be "recovered" afterwards.

Although used in clinic over a long period of time for the treatment of allergic diseases, the effect of common mast cell stabilizers such as sodium cromoglycate and ketotifen on MRGPRX2 mediated reaction was not investigated before (309). Ketotifen acts as a histamine 1 receptor antagonist and is also described to stabilize mast cells (310). While cromolyn sodium is generally considered to be a mast cell stabilizer (311). We found decreased mast cell activation in mast cells stimulated via MRGPRX2 via substance P, suggesting that ketotifen might be considered for treatment of non-IgE mediated mast cell driven inflammation besides typical IgE-mediated mast cell diseases.

Admittedly, our study has some limitations. Mast cell degranulation was only assessed via flow cytometry. Additional analyses such as β-hexosaminidase assay or measurement of released mediators such as cytokines and lipid mediator could be used to confirm our obtained results or even discriminate between IgE mediated or MRGPRX2 mediated mast cell activation. However, CD63 upregulation is a standardized and accepted method in literature to measure mast cell degranulation at single-cell level. In addition, variability in mast cell numbers and expression of surface receptors was observed. This might be explained by technical effects or inter-donor variance. These quality and quantity issues represent real-life situations and as

such, variation within our results was observed. Further experiments should take this into account this quantitative and qualitative variation.

In conclusion, primary human mast cell differentiation starting from buffy coats or fresh blood samples was optimized to obtain optimal MRGPRX2-expression. Obtained mast cells were functional for both IgE- and MRGPRX2- mediated activation, enabling research on MRGPRX2 dependent mast cell activation.

5. Supplementary material

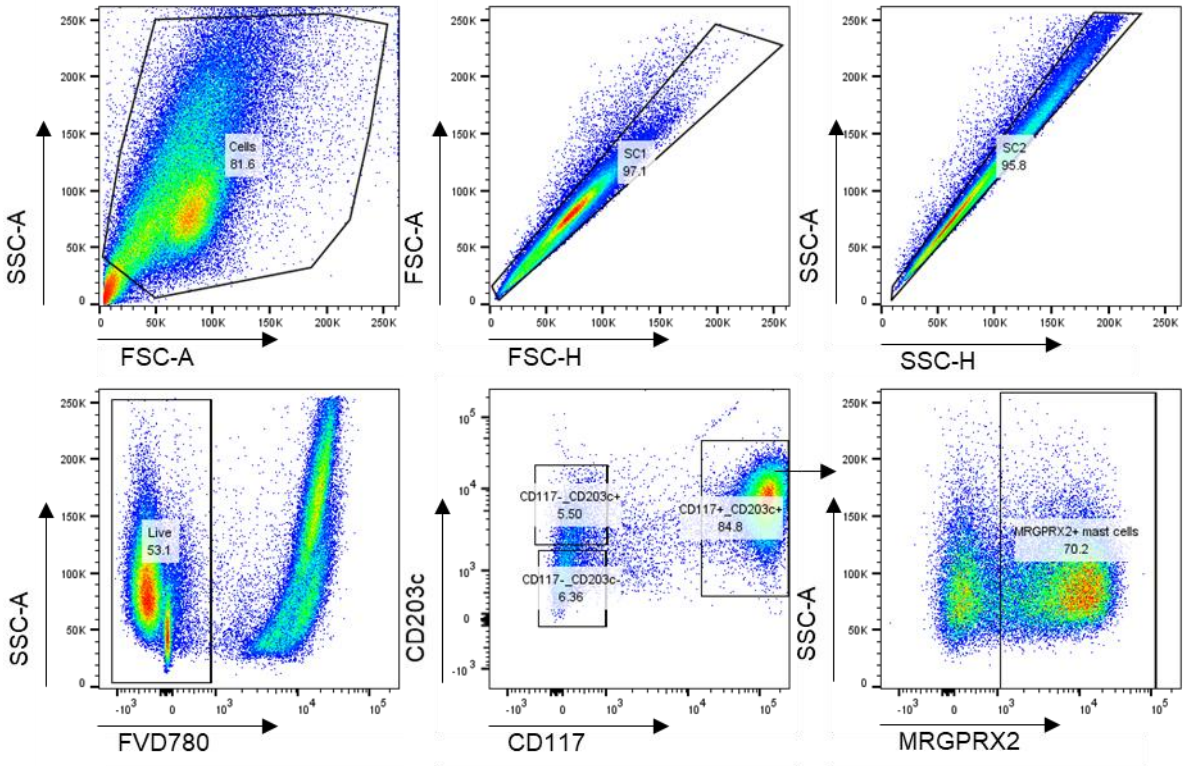

Figure E1: Representative gating strategy for cultured mast cells. (Applied on differentiated cells starting from peripheral blood sample)

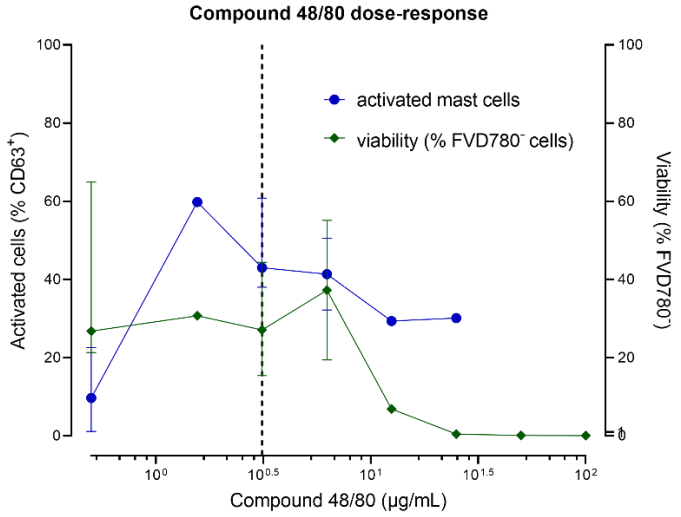

Figure E2: Non-IgE mediated activation of mast cells via C48/80 (n=4). Viability (% FVD780) is expressed on second x-axis.

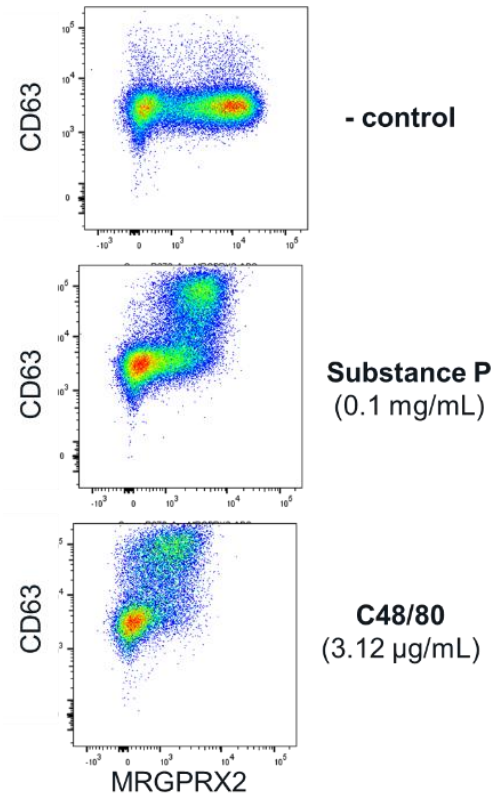

Figure E3: Representative plot demonstrating mast cell activation in MRGPRX2 expressing mast cells. (Applied on differentiated cells starting from buffy coat)

Chapter 10

General discussion

Human airways are continuously exposed to external triggers in the air, inducing epithelial triggering and potential damage. In this PhD thesis, we investigated airway inflammation as a result of external triggers inducing bronchoconstriction. In the first part, atopic state and exercise-induced bronchoconstriction (EIB) were studied in adolescent athletes, who are in this thesis defined as persons (12-18 years) who are trained in sports that requires physical activity (≥ 12 hours of sport a week). The second part included an in-depth analysis of exposure to external triggers on the airways of both early-career elite athletes and asthmatic patients. In the last part, the involvement of mast cells in non-allergic asthma, as potential targets for external stimuli induced epithelial cell cytokine production, was investigated.

1. Atopy and EIB in young athletes

1.1. Summary of the findings

A high prevalence of respiratory allergies, asthma and EIB has been reported amongst athletes (114, 187). Therefore, we studied atopic state and EIB in a population of early-career adolescent athletes (12 – 18 years). In chapter 4, we focused on early-career elite athletes (n=90), performing a high level of intense exercise at 'Flemish Elite Sports' Schools'. We found a sensitisation rate of 37%. Both AQUA© score (≥ 6) and FeNO levels (≥ 15 ppb) were able to predict atopy in this population (figure 1). In addition, we demonstrated that FeNO levels had higher sensitivity to predict atopy with multiple sensitisations. We were able to validate these findings in a cohort of recreational adolescent athletes (n=327) also performing at least 12 hours of physical exercise a week but not needed to be affiliated to an 'Elite Sports' School', in chapter 5 (figure 1). In this cross-sectional study, 40% of athletes were atopic, 14% reported a physician-based asthma diagnosis and 22% tested positive for EIB. An asthma diagnosis was found to be significantly associated with outdoor sport disciplines. Of these athletes with a positive EVH test, 80% of athletes were not treated with any inhalation therapy and 76% did not receive a prior asthma diagnosis, which term is often interchangeably used in real-life practice, indicating that there is a need to better identify athletes at risk for EIB. Wheezing during exercise was found to be an independent predictor for EIB in athletes without previous asthma diagnosis, while FeNO measurement was an independent predictor in the whole group and was specifically associated with EVH response in atopic athletes. A high sensitivity to predict EIB in athletes was found for AQUA© score of ≥ 6. Finally, measurement of serum

markers of epithelial damage showed association with intense training years, training type and severity of EIB.

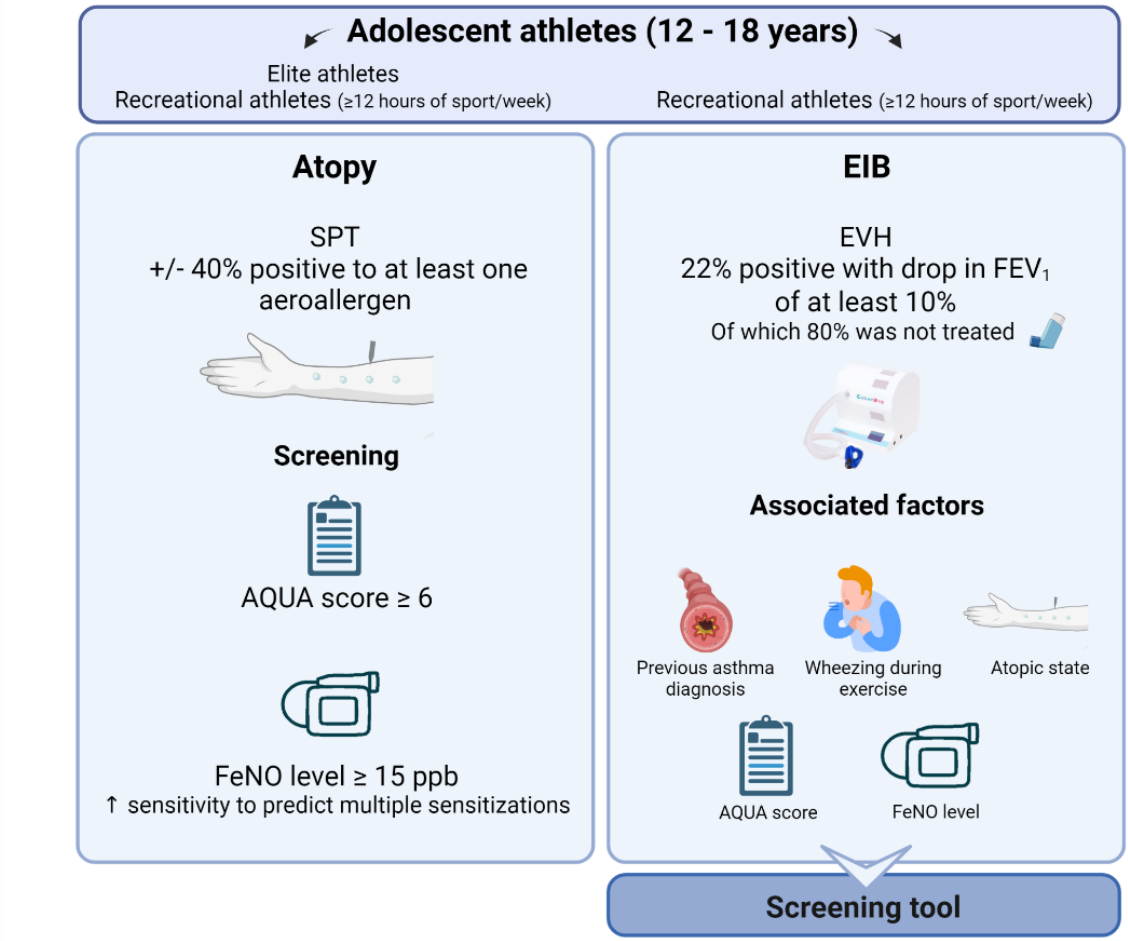

Figure 1: Screening possibilities for atopy and EIB in adolescent athletes. (Created with Biorender.com) AQUA, allergy questionnaire for athletes; EIB, exercise-induced bronchoconstriction; EVH, eucapnic voluntary hyperventilation; SPT, skin prick test. (Created with Biorender.com)

1.2. Relevance and limitation

Diagnosis of EIB is particularly relevant because of potential implications on performance in training and competition as airway narrowing during exercise compromises ventilatory capacity and efficiency (312). In addition, asthma diagnosis has been pointed towards risk factor for deaths in athletes associated with a sport activity (313). We identified that most of the EIB^{+} athletes did not have prior diagnosis or treatment, indicating that there is a need for a simple and robust clinical tool to aid in evaluating airway health in athletic individuals. The association of asthma with outdoor sport disciplines in our recreational athletes underlines the importance of the environment in which adolescent athletes exercise. Performing sport activities is suggested to increase the exposure to external triggers such as aeroallergens and air pollutants. However, we did not find a significant association with type of training and EIB

within this cohort of recreational athletes. This might be explained by the fact that these are recreational athletes, not performing sport activities on the highest level. The median training hours a week for recreational athletes was 14 hours/ week, while for elite athletes this was 20 hours of sport/week. We hypothesize that when training hours increase, a shift will occur toward harmful effects of exercise, which might be associated to environmental triggers or due to lack of power. The airway epithelium may play an important role in the pathogenesis in EIB (93). We indeed found higher markers of epithelial stress in athletes with a longer history of training years. In addition, we found increased epithelial damage in athletes with moderate EIB in comparison with athletes with mild EIB. In contrast, we were not able to discriminate EIB^- and EIB^+ athletes based on measured serum markers. In this study, we specifically focused on serum markers, as they are easy in daily use compared with sputum samples.

Regarding atopy, we found in both cohorts of athletes, including early-career and recreational athletes, similar presence of atopy, 37% and 40%, respectively, which is slightly higher than the general Belgian population (195) but in line with other authors reporting atopic state in adolescent athletes (136, 156). As atopy is frequently unrecognized and underreported, a screening tool may be relevant for athletes. The AQUA© questionnaire is a simple and easy-to-use tool to identify athletes who require further allergy testing and was validated in adolescent athletes. Based on our results we can conclude that FeNO levels are associated to atopy in adolescent athletes. Even in adolescent athletes with asthma, we found that FeNO (≥ 15 ppb) is a good and significant predictor for atopy. In literature, FeNO is considered to be a non-invasive marker of childhood asthma (191), which is typically characterized by Type 2 inflammation. For athletes, we hypothesize that FeNO is rather a marker of Type 2 inflammation present in atopic athletes, than a specific marker of asthma. Twenty-five percent of recreational athletes had suggested non-Type 2 asthma without increased FeNO levels. Furthermore, EIB diagnosis was significantly associated with atopy and FeNO levels negatively correlated with EVH response in atopic athletes, suggesting that higher levels of type 2 inflammation are associated with increased airway hyperreactivity. Therefore, we propose that FeNO can be a useful marker to detect EIB in atopic athletes. A major disadvantage of FeNO is that the measured levels are affected by other factors such as age, gender, height, smoking state and diet (314).

In literature it is suggested that atopy is a risk factor for bronchial hyperreactivity and EIB (93, 189). We also found a significantly higher proportion of atopic athletes within EIB^+ athletes compared with EIB^- athletes and an AQUA© score ≥ 6 had highest sensitivity to detect EIB. As expected, wheezing during exercise was found to have high specificity for EIB (205). Most published research on FeNO and EIB focus on EIB in children and adolescents with asthma (315–317). However, we also demonstrated its role in association with EIB in atopic recreational athletes specifically. The heterogenous composition of athletes with and without asthma limits the development of a uniform screening test. We now applied obtained results of all athletes in a suggested flowchart to screen adolescent athletes performing at least 10 hours of sport a week, containing 'wheezing during exercise' and AQUA© score ≥ 6 or FeNO ≥ 15 ppb, followed by atopy testing. The results of allergy testing can be of added value for the athlete as exposure to specific triggers can be avoided during training. This heterogenous cohort of recreational athletes also highlights the complex overlap between EIB and asthma in athletes. However, it reflects the real-life situation in which a screening tool for EIB might be applied. It is hypothesised that EIB may evolve into asthma. Up to now, there are no reports on the progression of asymptomatic EIB and whether it may evolve into EIB with symptoms or even asthma. A positive EVH test in asthmatic athlete might point towards uncontrolled asthma. Our study is limited as we focused on diagnosis of EIB and did not perform a diagnostic test for asthma (e.g. baseline reversibility test) or other differential diagnosis. As such, undiagnosed asthma may be present in the athletes.

1.3. Future perspectives

Our results support the importance to better monitor allergic and respiratory conditions in adolescent athletes. For atopy, we were able to show and validate the accuracy of AQUA© and FeNO. These tools might be useful to emphasize the high prevalence of allergy in athletes and might provide the first step towards implementing flowcharts for allergy diagnosis and management in sports medicine. Also, a greater awareness of EIB among adolescent is needed. More research is necessary to better understand the underlying mechanisms of EIB in athletes. Markers of epithelial damage might be candidates to fill this gap. In order to implement the suggested flowchart, we have to validate these results in an independent cohort of athletes sufficiently powered to allow screening at the Flemish or national level. A cost-benefit analysis is necessary to see whether the suggested flowchart is feasible or

adaptations regarding sensitivity and specificity are needed. In addition, we need to study whether early treatment of subjects with positive EVH test results in reduced prevalence of EIB during follow-up. By implementing a flowchart to identify athletes at risk for EIB, we highly advise that a clinical guideline committee develops guidelines on how these athletes should be managed throughout their sports careers.

2. In-depth analysis of exposure to external triggers on the airways

2.1. Summary of the findings

EIB is highly prevalent in elite athletes and seems to be associated with environmental exposures during training. Moreover, environmental exposures are important triggers for exacerbations and respiratory symptoms in asthmatic patients. To study the effect of external exposure on the airways of elite athletes and asthmatic patients, we performed RNA-Seq on induced sputum samples. An overview of suggested pathways involved in respiratory response to external triggers based on obtained results are summarised in figure 2. In chapter 6, we investigated the effect of intense exercise and associated exposure to air pollution on the airways of adolescent elite athletes (n=90) compared with healthy controls (n=25). Our results showed increased carbon load in sputum macrophages in athletes compared with control subjects performing <6 hours of sport a week. In addition, genes related to epithelial damage, airway inflammation and immune trafficking were upregulated in athletes' sputum samples compared with control subjects. Athletes had significantly higher substance P and calprotectin levels as well as IL-6, IL-8, TNF-α mRNA levels compared with controls. Based on a preliminary RNA-Seq analysis between atopic EIB^+ and EIB^- athletes, increased pathways for oxidative signalling and neuroinflammation were found for EIB^+ compared to EIB^- athletes. The maximal fall in FEV_1(%) after EVH test in athletes was significantly associated with prior PM_{10}.

The effect of external triggers on airways of asthmatic patients was studied in chapter 7. A retrospective analysis in patients' files searching for environmental exposures was performed. Firstly, increased levels of alarmins were found in asthmatics compared with healthy controls, pointing towards epithelial damage. Exposure to cigarette smoke was investigated, as it may alter the type of immune response and cytokine production in airway inflammation (265). RNA-Seq analysis revealed upregulation of CYP1B1 and downregulation of CLDN4, ECM1 and TJP1 in active (n=8) compared to never smoking asthma patients (n=13). Even the comparison

of never (n=13) versus former smokers (n=20) resulted in differentially expressed genes (DEGs), which were all downregulated in former smokers. These genes were related to epithelium development and filament cytoskeleton organization. Next, work-related exposures to irritants was investigated. We found that serum SP-D levels were significantly elevated in asthmatic patients who reported exposure to cleaning products compared with patients who did not report any exposure, pointing towards epithelial damage. Based on RNA-Seq analysis, the aryl hydrocarbon receptor (AhR) signalling pathway was considered to be important. In addition, asthmatics exposed to cleaning products were significantly associated to IL-22-high cytokine profile.

2.2. Relevance and limitation

Sputum induction is an important non-invasive way to study lower airways. Transcriptomic analysis of these samples by next generation sequencing enables high throughput and thorough characterisation of gene expression profiles. However, not every subject is able to produce a useful sputum sample. Furthermore, the quality necessary to perform RNA-Seq is not reached by every subject, especially in healthy controls, resulting in a reduced usable number of samples. Unfortunately, swimmers as a sport discipline did not consent to produce a sputum sample for the cross-sectional study due to complaints associated with inhalation of hypertonic saline in a previous study performed by our research group (101). The fact that especially swimmers had these complaints when compared to other disciplines and in this swimmers group a high proportion of EIB^{+} subjects was found, suggests that the airways of swimmers might already be more “sensitive”. The elevated levels of serum CC16 in swimmers compared to other sport disciplines supports this hypothesis (chapter 5).

The relationship between air pollution, EIB and elite sports has not been extensively studied. However, the negative effect of air pollution on human airways have been demonstrated (109). This can be of importance in high traffic areas such as Flanders. The increase in black carbon load of airway macrophages in athletes compared with controls is compatible with the hypothesis that the increased inhalation rate during exercise increases the inhalation dose of air pollutants. Unexpectedly, we did not find a difference in carbon load between athletes performing in- or outdoor sport disciplines. This might be explained by poor air cleaning in ventilation systems of sports halls.

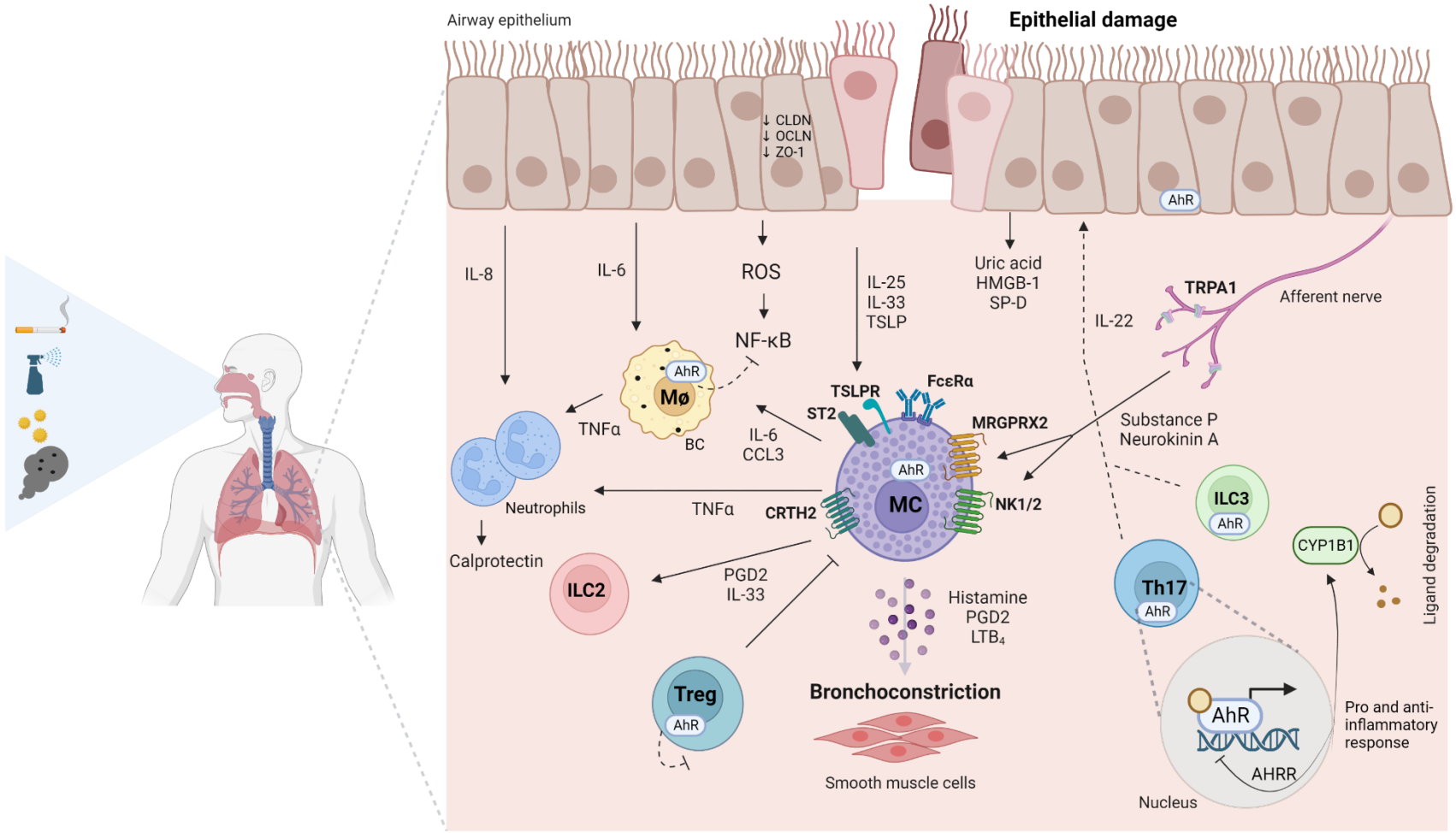

Figure 2: Exposure to external triggers: central role for mast cell? (Created with Biorender.com) Exposure to external triggers investigated within this thesis: cigarette smoke, cleaning products, allergens and air pollution can induce epithelial damage. This might lead to decrease in tight junctions (CLDN, OCLN, ZO-1), the release of DAMPS (Uric acid, HMGB-1, SP-D), alarmins (IL-33, TSLP) and cytokines (IL-6, IL-8, IL25) by epithelium. These will initiate immune response. Furthermore, afferent nerves can be triggered resulting in the release of neuro-mediators (substance P and neurokinin A). Furthermore, external triggers might also generate reactive oxygen species (ROS), which can activate NF-κB mediated immune response. Mast cells can be activated via IgE mediated and non-IgE mediated pathways, resulting in degranulation and bronchoconstriction. The aryl hydrocarbon receptor is expressed within different immune cells. Its effect is indicated with dotted line. (Created with Biorender.com)

Besides, we are not aware of the number of hours of sport activities that is performed individually inside or outside, as athletes performing an indoor sport like volleyball or basketball may also train outside. In addition, we found significantly lower levels of OCLN and ZO-1 mRNA levels in athletes exposed to higher levels of PM_{10} and also their airway response to EVH test was associated with prior PM_{10} exposure. These results highlight the association of exposure to air pollution and airway hyperreactivity in adolescent elite athletes. Our study provides further evidence that action should be taken to reduce PM in general and improve air quality during training sessions and sport competitions in particular.

Several mechanisms underlying EIB have been proposed including thermal and osmotic hypothesis, neurogenic inflammation and epithelial damage (93). Our exploratory work on EIB^+ athletes suggested oxidative stress as underlying mechanism. Indeed, elite sport activities have already been described as stimulus for oxidative stress (262), but we suggest a link with EIB. We furthermore suggested activation of the neuroinflammatory pathway in EIB^+ athletes. These results should be considered as preliminary as they are based on the comparison of 2 EIB^+ atopic athletes compared with 4 matched EIB^- atopic athletes. Neuropeptides such as substance P and neurokinin-A (NKA) are also described by others to be involved with EIB (93). As suggested in chapter 8, neuro-mediators might be involved in the activation of mast cells, releasing mediators involved in bronchoconstriction such as cysLTs and PGD2.

More athletes could be included in the RNA-Seq analysis comparing sputum RNA data from elite athletes (n=37) with healthy controls (n=11). We confirmed pro-inflammatory reactions in sputum transcriptome of elite athletes and validated these results by increased sputum mRNA levels of IL-6, IL-8 and TNF-α. Similarly, to other reports, we found epithelial damage measured by elevated HMGB-1 and downregulation of CLDN15, a component of the tight junctions. In contrast to other reports, we also focus on potential beneficial effects in elite athletes compared to controls. Specifically, we found an upregulation of INF-γ response, which is considered to be an anti-inflammatory cytokine. In addition, a downregulation of TLR3 was observed, which might be beneficial due to reduced inflammatory capacity of leukocytes, limiting chronic inflammation.

Although avoiding of environmental factors is recommended in the management of asthma, the effect of environmental exposure to irritants in cleaning products on asthma control is

limited. Based on a study with a large population size, it was demonstrated that regular use of disinfectants and cleaning products may contribute to poor asthma control in elderly woman (318). Regardless of the limitations of our study, this is the first study performing an in depth-analysis of the sputum transcriptome of asthmatic patients in association to the exposure to cleaning products. The AhR signalling pathway seem to be involved in the reaction to exposure to cleaning products. AhR is ligand-activated transcription factor effecting mast cells, B cells, macrophages, Th17 and Tregs, all of them influencing airway inflammation (319)(figure 2). AhR activation will induce cytochrome P4502 (CYP1) enzymes, which oxygenate the AhR ligand, leading to their metabolic clearance and detoxification (320). AhR activation will stimulate IL-22 production (274). We found a significant association between IL-22-high cytokine profile, based on 90th percentile of mRNA levels of controls, and asthmatics exposed to cleaning products. In contrast to mice, no IL-17 high cytokine was found in these asthmatics (321). Investigating the sputum transcriptome of smoking asthmatic patients, also an upregulation of the AhR pathway including CYP1 was found. This suggest a similar reaction of the airways to xenobiotic stimuli. Cigarette smoking has adverse health effects including reduction of lung function, amplified risk on pathogen invasion by disrupting epithelial barrier and immunomodulatory properties (322–324). Despite the fact that cigarette smoking is known to exacerbate asthma, only limited clinical asthma studies have been conducted involving smokers. We indeed confirmed reduced epithelial tight junctions in smoking asthmatic patients (279, 324). Furthermore, a significant association between asthma in adolescent athletes and a household member who smokes was found in chapter 5, which reconfirms the negative impact of smoking. An important limitation of the study in asthmatics is the retrospective study design, as only reported environmental exposures in the patient file were included. The results for work-related exposure to cleaning products was based on a small group of six patients, which were all female. This might bias the results of RNA-Seq analysis.

2.3. Future perspectives and recommendations

The cross-sectional and retrospective design of both studies do not permit causal relations to be drawn. Both studies are considered to be observational. Therefore, exposition studies in mice models might be relevant to study the impact a specific trigger and as such unravel the similarities and differences between bronchoconstriction induced by irritants and induced by

a repeated high ventilatory burden as in endurance trained athletes. As we found in both cases signs of epithelial damage. Regarding the underlying mechanisms of EIB, further research may focus on the neuro-immune response. Transient receptor potential ankyrin 1 (TRPA1) might be involved as irritants may activate TRPA1 (96), inducing the release of substance P and NKA by C-fibers, which promote inflammatory response. In addition, more research into the oxidative stress pathways could increase our knowledge on the underlying mechanisms of EIB. Due to the temporary nature of reactive oxygen species, this can be assessed indirectly via measuring levels of DNA/RNA damage, lipid peroxidation and protein oxidation/nitration. Serum oxidative stress markers were recently shown to be higher in power sports (such as cycling and long distance athletics), which may alter tissue healing capacity (325). This might explain the disruption of tissue homeostasis and transition to hyperreactivity of the airways seen in EIB.

Variability in the results of EVH test has been repeatedly reported. Also the recent position paper of European Academy of Allergy and Clinical Immunology (EAACI) highlights the importance of repeat assessments taking into account environmental conditions (202). Indeed, external factors could perhaps influence those assessments. We indeed found a significant negative association between the maximal fall in FEV_1 and prior PM exposure. This observation shows that also air pollution should be considered as an important external trigger inducing airway reactivity. A prospective study design is warranted to further examine the link with exposures in EIB and/or asthma. In such further research, personal samplers can be used to better track exact exposure to air pollutants or even aero-allergens of individual athletes.

PM is emitted from incomplete combustion processes, predominantly from traffic exhaust or industrial emissions including power production. As Flanders is a densely-populated region with high traffic density (figure 3), we suggest to focus on transport sector to reduce PM levels by limiting use of diesel cars (lacking soot filters), making electric cars financially accessible to a larger population, expanding low emission-zones in city centres, improving public transport but also by education and increasing awareness of effects of air pollution. For elite athletes it is suggested to avoid prolonged high intensity endurance exercise in highly polluted periods to decrease personal exposure (326). For example, in case of elevated pollution levels, trainings can be scheduled in the morning or evening as pollution levels are generally lower

during these periods. As applied by the IOC (327), an expert committee can monitor air quality, give updates and make recommendations. Furthermore, there is an important role for medical experts to ensure that an athlete is aware of risks associated with air pollution exposure.

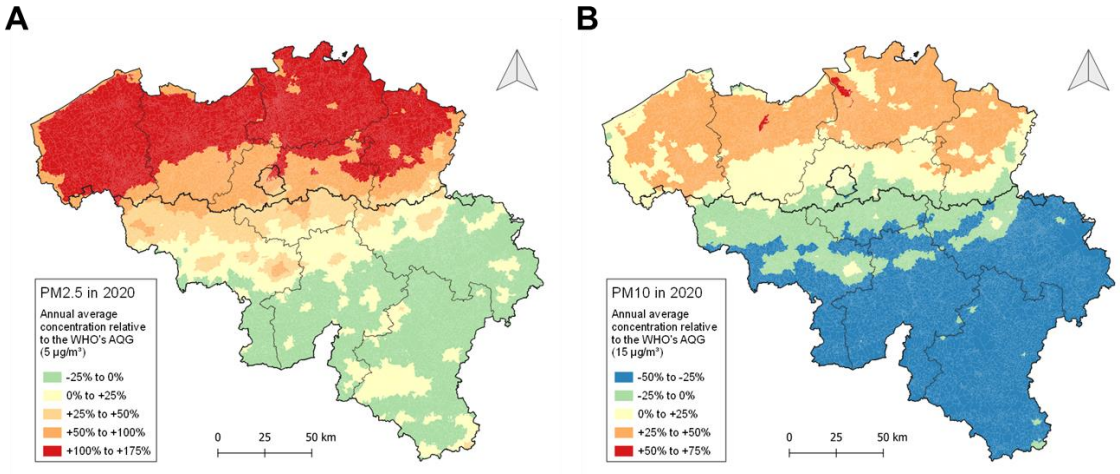

Figure 3: Relative concentration of PM2.5 and PM10 in Belgium, 2020. (Source: for a healthy Belgium (328)). AQG, air quality guidelines; PM_{10}, particulate matter ≤ 10 µm; $PM_{2.5}$, particulate matter ≤ 2.5 µm; WHO, world health organization.

In further studies, one can specifically focus on work-related exposure to cleaning products in comparison to a matched control population. The use of mice models might also help to investigate the role of AhR in regulation asthmatic phenotype. For example, an AhR deficient mice model demonstrated reduced airway hyperresponsiveness in an irritant-induced asthma model (with chlorine)(329). Limiting the use of disinfectants and exposure to cleaning products may help improve asthma control. Specifically, one might avoid strong chemicals that can induce respiratory irritation and ensure adequate ventilation when using cleaning products. This helps to reduce the concentration of cleaning product fumes in the air.

3. Mast cell in non-allergic asthma

3.1. Summary of the findings

Limited knowledge is available on the role of mast cell in non-allergic asthma. In this part, we investigated the role of mast cell in non-allergic asthma using an *ex vivo* and *in vitro* approach. In chapter 8**,** we developed a flow cytometric panel to characterize mast cells in induced sputum samples of asthmatic patients and healthy controls. In this pilot study, we found significantly higher mast cell percentages in allergic asthma compared to healthy controls. However, for non-allergic asthma patients also mast cell activation was observed. Sputum mast cells correlated with NKA levels and were associated to lower lung function. We were able to detect MRGPRX2 expression on mast cells, but this was however not associated with

an allergic or non-allergic asthma phenotype. To better investigate the role of MRGPRX2 receptor on human mast cells, an *in vitro* differentiation model of human mast cells expressing MRGPRX2 was optimized in chapter 9. $CD34^+$ progenitor cells of peripheral blood or buffy coat were cultured in a serum-free methylcellulose based medium for 4 weeks. FBS addition significantly reduced and IL-6 increased MRGPRX2 expression, resulting in functional mast cells. In order to study mast cell functionality, both IgE- mediated (anti-IgE) and non-IgE mediated stimuli (substance P and C48/80) were added, all of them resulting in mast cell activation observed by increased CD63 expression. Also inhibition assays were performed showing the ability of ketotifen to inhibit MRGPRX2-mediated activation in contrast to cromolyn sodium.

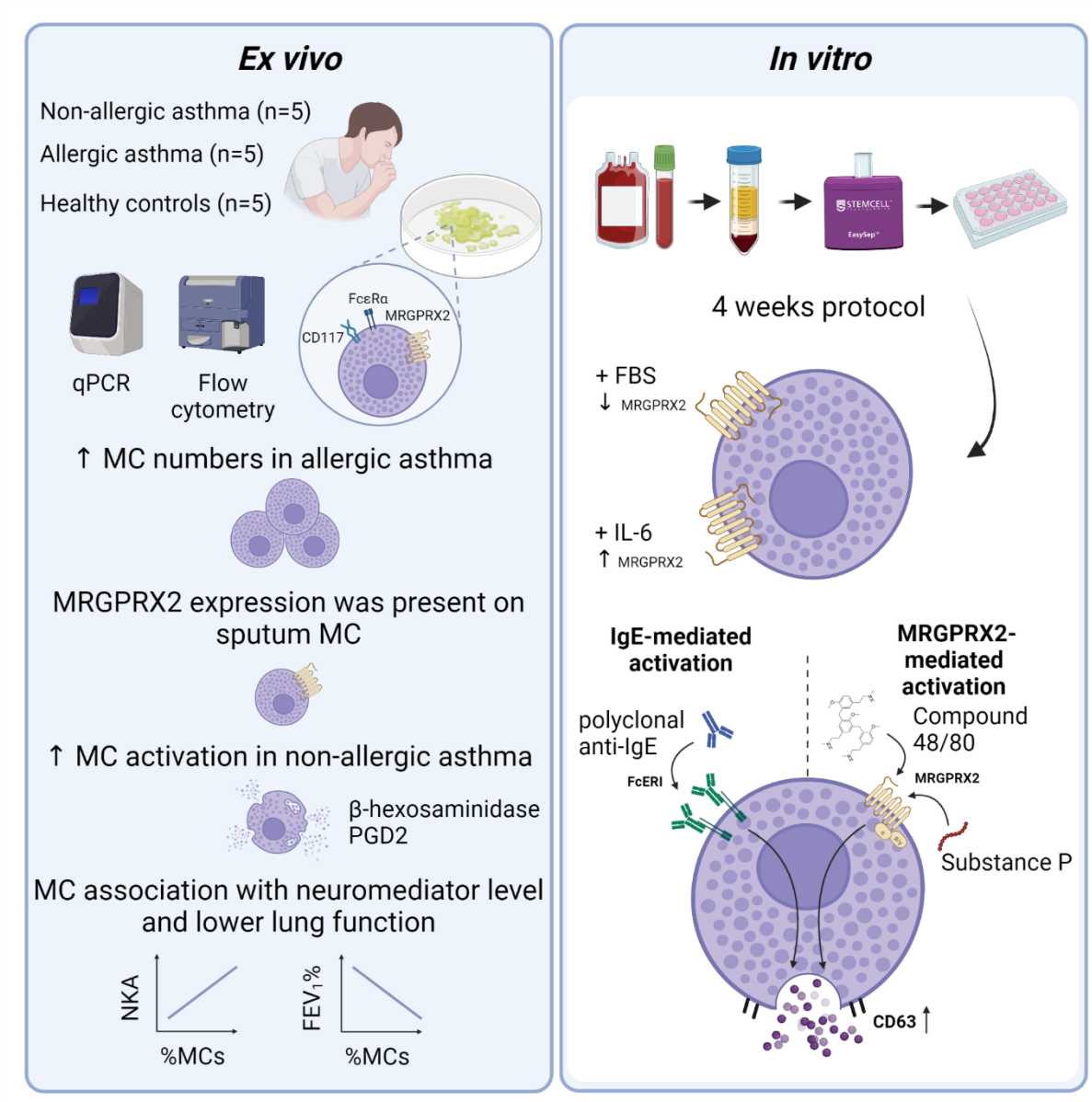

Figure 4: *Ex vivo* and *in vitro* approach to investigate MRGPRX2 expressing mast cells. MC, mast cell; MRGPRX2, Mas related G protein coupled receptor family member X2; NKA, neurokinin-A (Created with Biorender.com)

3.2. Relevance and limitation

By our knowledge only one recent study investigated mast cells via flow cytometry in induced sputum samples (290). Similarly, mast cells were elevated in asthma and were associated to poorer lung function. Furthermore, we found mast cell activation in non-allergic asthma, suggesting that mast cells can play a role in airway inflammation of both allergic and non-allergic asthma phenotypes. The low sample size in this pilot study is an important limitation. However, these results can be used as basis for new research. Our pilot study suggested namely the involvement of mast cells in neuro-immune reaction. It is known that mast cells can be activated via neuro-mediators. The most studied mediator of neural communication with mast cells is substance P, which can activate mast cells via classical NK-1 receptor, but also via MRGPRX2 receptor (330). However, the role of MRGPRX2 in response to other neuropeptides such as NKA is not known yet. As human MRGPRX2 receptors are generally more sensitive to substance P compared with the mouse orthologue, MrgprB2, a human *in vitro* human mast cell model can be more useful to study optimal mast cell influencing triggers/compounds (49).

The development of a human mast cell *in vitro* culture creates multiple opportunities to investigate both IgE-dependent and independent activation of mast cells. Our optimized protocol showed different advantages compared to previous published protocols. Many published protocols require at least 6 to 12 weeks to produce mature mast cells, which make them laborious and time consuming (83, 331–333). Within our results, a time-frame of 4 weeks was ideal to obtain viable mature tryptase and chymase containing mast cells. Next, we were able to obtain differentiated mast cells from both buffy coat and peripheral blood, which enables further research to obtain mast cells from characterized patient/control populations. Moreover, our obtained mast cells were characterized by flow cytometry and their functionality was assessed via activation and inhibition assays, with specific focus on non-IgE mediated activation via MRGPRX2. Nonetheless, different yields of mast cells were obtained while using the same differentiation conditions, for which we have no explanation. Inter-donor and technical variations are possible suggestions. In addition, mast cell activation was only assessed via CD63 upregulation and were not measured via mediator release tests such as histamine or β-hexosaminidase. However, flowy cytometry enables to study mast cell activation on a single cell level.

3.3. Future perspectives

The optimized flow cytometry panel can be used to identify and characterize mast cells in sputum samples of more extensive cohorts. Furthermore, future research may focus on IgE-independent mast cell activation in both allergic and non-allergic asthma. Specially, one can focus on the neuro-immune cross talk as neuro-mediators can also activate mast cells. The optimized differentiation protocol for mast cells can be used to set up co-cultures with epithelial cells and innate lymphoid cells to unravel the interaction between these cell types. A first step to develop these co-cultures would be to transfer the medium of activated mast cells to epithelial cells and ILCs. In a second co-culture system, mast cell can be cultured in the lower chamber of a transwell plate. Cultured epithelial cells or ILCs can be added to the upper chamber. Finally, mast cells can be placed directly with another cell type. However, we demonstrated that mast cells are very sensitive to changing local environment, as documented by the fact that addition of IL-6 increases the expression of MRGPRX2 while it decreases upon addition of FBS. This can be of importance as Calu-3 cells (immortalized bronchial epithelial cell line) are routinely grown in medium supplemented with 10% FBS (334). In the same way, isolated ILCs are normally cultured in culture medium supplemented with 10% FBS and cytokines IL-2 and IL-7 (335, 336). In a next step of experiments, the effect of alarmins and DAMPs can be added to simulation experiments in order to see if these environmental factors might influence mast cell response or receptor expression ine one sense or another. In addition, these differentiated mast cells can be used to screen potential drugs targeting mast cells. With the advantage of these differentiated mast cells that they are functional via IgE mediated as well as non-IgE mediated pathway. Therefore, we hope that both our *ex vivo* and *in vitro* techniques might help to further clarify the role of mast cells in non-IgE mediated asthma pathogenesis.

4. Covid-19 Pandemic

The COVID-19 pandemic had a clear impact on this thesis. First of all, study visits were limited in the hospital during the different COVID-19 peaks (March-June 2020, November-December 2020, …) and patients were not always eager to come to the hospital only for study purpose (without medical need). In addition, access to organized sports activities in the age group of 12-18 years was limited in Belgium between March 2020 till June 2021. The inclusion of young athletes performing at least 12 hours was an important inclusion criterion in the screening

study for EIB (chapter 5), so inclusion was highly influenced. Also inclusion of patients with asthma for mast cell characterisation (chapter 8) was hampered due to the limited study visits possible in these periods and the restriction on use of sputum induction because of aerosolization (March-June 2020). No other techniques were available to collect samples of the lower airways in a non-invasive manner. Finally, the protocol for the performance of sputum induction after its re-allowance was extended (e.g. use of specific enclosed room with continuous extraction hood, gloves, isolation gown, shield and FFP2 mask) to protect both patients and technician, and was as such more time consuming. Therefore, chapter 8 was restricted to a pilot study. Despite these limitations, also potential opportunities for future research related to this thesis appeared, as following questions arose: Can EIB disappear if an adolescent athlete stop with intense exercise? How long will it take until epithelial damage caused by external triggers is gone? But also, will we detect less airway hyperreactivity when there is less air pollution? As during Covid-19 pandemic there was less traffic and accordingly less air pollution present but comparison with baseline levels from before the pandemic was difficult, as most subjects were followed longitudinally.

5. General conclusion

In our study within adolescent intense athletes (12 – 18 years), we have observed a high sensitisation rate up to 40%. An AQUA© score ≥ 6 and FeNO level ≥ 15 ppb were validated to predict atopy. As atopy is frequently underreported and unrecognized in athletes, the easy collectable and low-cost results of AQUA© and/or FeNO are relevant for screening in adolescent athletes. Indeed, as atopy is a risk factor for EIB, EIB might also be underreported. EIB was found in 22% of recreational intense athletes, of which 76% did not have a prior diagnosis. Our results indeed point to the underdetection of EIB in adolescent athletes, once more confirming the need for a screening test for EIB. We suggested a screening tool containing 'wheezing during exercise' and 'AQUA© score ≥ 6' ready to be implemented. Further studies in an independent cohort of athletes including cost-benefit analysis are needed to implement this screening tool for its use within the current organised medical screenings for athletes. Furthermore, the association of asthma especially with outdoor sport disciplines underlines the potential impact of the environment in which intense exercise is performed.

Moreover, our research has focused more in depth on the effect of specific external triggers on airways of elite athletes and asthmatics. Black carbon load in airway macrophages was significantly increased in elite athletes compared with controls. This is consistent with our hypothesis that the increased ventilation rate during intense exercise is associated with increased exposure to external triggers. As increased hyperreactivity was found in athletes exposed to higher PM levels, there is a clear need to improve air quality in Flanders. Also asthmatics are considered to be more vulnerable to external triggers, which was demonstrated by increased levels of epithelial damage. For both, asthmatics and elite athletes, an important role for the epithelial barrier in homeostasis as well as in activation after triggering/damage was found, being the first line of defence. Indeed, the sputum transcriptomics was significantly different in asthmatics exposed to external irritants such as cigarette smoke and work-related exposure to cleaning products compared with asthmatics without such exposures. The aryl hydrocarbon receptor (AhR), which is expressed in multiple immune cells seemed to be an important player in this response to external triggers. This highlights the complex role that AhR may have in the development and progression of asthma, modulating the immune response in reaction to environmental stimuli.

Finally, our research focussed on mast cells, responding to epithelial cytokines, resulting in bronchoconstriction. In this thesis, we developed an *ex vivo* model, investigating the mast cell expressing MRGPRX2 in induced sputum samples and in an *in vitro* model, especially developed to investigate non-IgE mediated activation presumably via MRGPRX2. We believe that our results are an important step towards a revised role of the 'old mast cell' with typical IgE-mediated activation towards a non-IgE mediated role in both allergic and non-allergic airway inflammation. We hypothesize that MRGPRX2 expressing mast cells are important in the neuro-immune interaction. Our optimized differentiation protocol might be a useful tool to explore this role of mast cell *in vitro.*

Taken together, external triggers have potential harmful effects on airway barrier. In intense adolescent athletes an elevated impact is suggested due to increased ventilation rate. Therefore, proper follow-up (including screening strategies for atopy and EIB) and improvement of environmental conditions are recommended. The revised role of the mast cells might result in new targets of asthma therapy.

References

1. Weitnauer M, Mijošek V, Dalpke AH. Control of local immunity by airway epithelial cells. *Mucosal Immunol* 2016;9:287–298.
2. Treuting PM, Dintzis SM, Montine KS. *Comparative Anatomy And Histology: A Mouse, Rat, And Human Atlas Second Edition*. Mica Haley; 2017.
3. Gibson PG, Simpson JL. The overlap syndrome of asthma and COPD: what are its features and how important is it? *Thorax* 2009;64:728–735.
4. Labaki WW, Han MK. Chronic respiratory diseases: a global view. *Lancet Respir* 2020;8:531–533.
5. Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention. 2022;at <https://ginasthma.org/>.
6. WHO. Chronic respiratory diseases: asthma. at <https://www.who.int/news-room/questions-and-answers/item/chronic-respiratory-diseases-asthma>.
7. Lemanske RF, Busse WW. Asthma: Clinical expression and molecular mechanisms. *J Allergy Clin Immunol* 2010;125:95–102.
8. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 2012;18:716–725.
9. Hoshino M, Morita S, Iwashita H, Sagiya Y, Nagi T, Nakanishi A, Ashida Y, Nishimura O, Fujisawa Y, Fujino M. Increased expression of the human Ca2+-activated Cl- channel 1 (CaCC1) gene in the asthmatic airway. *Am J Respir Crit Care Med* 2002;165:1132–6.
10. Ntontsi P, Photiades A, Zervas E, Xanthou G, Samitas K. Molecular Sciences Genetics and Epigenetics in Asthma. *Genet Epigenetics Asthma Int J Mol Sci* 2021;2021:2412.
11. Holgate ST, Wenzel S, Postma DS, Weiss ST, Renz H, Sly PD. Asthma. *Nat Rev Dis Prim* 2015;1:1–22.
12. Lambrecht BN, Hammad H, Fahy J V. The Cytokines of Asthma. *Immunity* 2019;50:975–991.
13. Jonckheere A-C, Bullens DMA, Seys SF. Innate lymphoid cells in asthma. *Curr Opin Allergy Clin Immunol* 2019;19:53–60.
14. Guia S, Fenis A, Vivier E, Narni-Mancinelli E. Activating and inhibitory receptors expressed on innate lymphoid cells. *Semin Immunopathol* 2018;40:331–341.
15. Sonnenberg GF, Hepworth MR. Functional interactions between innate lymphoid cells and adaptive immunity. *Nat Rev Immunol* 2019;19:599–613.
16. Kuruvilla ME, Lee FEH, Lee GB. Understanding Asthma Phenotypes, Endotypes, and Mechanisms of Disease. *Clin Rev Allergy Immunol* 2019;56:219.
17. Luo W, Hu J, Xu W, Dong J. Distinct spatial and temporal roles for Th1, Th2, and Th17 cells in asthma. *Front Immunol* 2022;13:4598.
18. Britt RD, Thompson MA, Sasse S, Pabelick CM, Gerber AN, Prakash YS. Th1 cytokines TNF-α and IFN-γ promote corticosteroid resistance in developing human airway smooth muscle. *Am J Physiol - Lung Cell Mol Physiol* 2019;316:L71.
19. Nair P, Surette MG, Virchow JC. Neutrophilic asthma: misconception or misnomer? *Lancet Respir Med* 2021;9:441–443.
20. Schleich FN, Manise M, Sele J, Henket M, Seidel L, Louis R. Distribution of sputum cellular phenotype in a large asthma cohort: predicting factors for eosinophilic vs neutrophilic inflammation. *BMC Pulm Med* 2013;13:11.
21. Manise M, Bakayoko B, Schleich F, Corhay JL, Louis R. IgE mediated sensitisation to aeroallergens in an asthmatic cohort: relationship with inflammatory phenotypes and disease severity. *Int J Clin Pract* 2016;70:596–605.
22. Brusselle GG, Maes T, Bracke KR. Eosinophils in the Spotlight: Eosinophilic airway inflammation in nonallergic asthma. *Nat Med* 2013;19:977–979.
23. Boonpiyathad T, Sözener ZC, Satitsuksanoa P, Akdis CA. Immunologic mechanisms in asthma. *Semin Immunol* 2019;46:101333.
24. GINA Main Report - Global Initiative for Asthma - GINA. at <https://ginasthma.org/gina-reports/>.
25. Kavanagh JE, Hearn AP, Jackson DJ. A pragmatic guide to choosing biologic therapies in severe asthma. *Breathe* 2021;17:1–15.
26. Hellings PW, Steelant B. Epithelial barriers in allergy and asthma. *J Allergy Clin Immunol* 2020;145:1499.
27. Hewitt RJ, Lloyd CM. Regulation of immune responses by the airway epithelial cell landscape. *Nat Rev Immunol* 2021;21:347–362.
28. Iwasaki A, Foxman EF, Molony RD. Early local immune defenses in the respiratory tract. *Nat Rev Immunol* 2017;17:7.
29. Ridley C, Thornton DJ. Mucins: the frontline defence of the lung. *Biochem Soc Trans* 2018;46:1099.
30. Tarran R, Grubb BR, Gatzy JT, Davis CW, Boucher RC. The relative roles of passive surface forces and active ion transport in the modulation of airway surface liquid volume and composition. *J Gen Physiol* 2001;118:223–236.
31. Nakagami Y, Silvio Favoreto J, Zhen G, Park S-W, Nguyenvu LT, Kuperman DA, Dolganov GM, Huang X, Boushey HA, Avila PC, Erle DJ. The Epithelial Anion Transporter Pendrin Is Induced by Allergy and Rhinovirus Infection, Regulates Airway Surface Liquid, and Increases Airway Reactivity and Inflammation in an Asthma Model. *J Immunol* 2008;181:2203.
32. Hohlfeld JM. The role of surfactant in asthma. *Respir Res* 2001;3:1–8.

33. Rezaee F, Georas SN. Breaking barriers: New insights into airway epithelial barrier function in health and disease. *Am J Respir Cell Mol Biol* 2014;50:857–869.
34. Carlier FM, de Fays C, Pilette C. Epithelial Barrier Dysfunction in Chronic Respiratory Diseases. *Front Physiol* 2021;12:1–27.
35. Parker D, Prince A. Innate Immunity in the Respiratory Epithelium. *Am J Respir Cell Mol Biol* 2011;45:189.
36. Lambrecht BN, Hammad H. The airway epithelium in asthma. 2012;18:684–692.
37. Müller I, Alt P, Rajan S, Schaller L, Geiger F, Dietrich A. Transient Receptor Potential (TRP) Channels in Airway Toxicity and Disease: An Update. *Cells 2022, Vol 11, Page 2907* 2022;11:2907.
38. Elieh Ali Komi D, Wöhrl S, Bielory L. Mast Cell Biology at Molecular Level: a Comprehensive Review. *Clin Rev Allergy Immunol* 2020;58:342–365.
39. Reber LL, Sibilano R, Mukai K, Galli SJ. Potential effector and immunoregulatory functions of mast cells in mucosal immunity. *Mucosal Immunol* 2015;8:444–463.
40. Bradding P, Arthur G. Mast cells in asthma - state of the art. *Clin Exp Allergy* 2016;46:194–263.
41. Dwyer DF, Ordovas-Montanes J, Allon SJ, Buchheit KM, Vukovic M, Derakhshan T, Feng C, Lai J, Hughes TK, Nyquist SK, Giannetti MP, Berger B, Bhattacharyya N, Roditi RE, Katz HR, Nawijn MC, Berg M, van den Berge M, Laidlaw TM, Shalek AK, Barrett NA, Boyce JA. Human airway mast cells proliferate and acquire distinct inflammation-driven phenotypes during type 2 inflammation. *Sci Immunol* 2021;6:.
42. Yu Y, Blokhuis BR, Garssen J. Non-IgE mediated mast cell activation. *Eur J Pharmacol* 2016;778:33–43.
43. Enoksson M, Möller-Westerberg C, Wicher G, Fallon PG, Forsberg-Nilsson K, Lunderius-Andersson C, Nilsson G. Intraperitoneal influx of neutrophils in response to IL-33 is mast cell-dependent. *Blood* 2013;121:530–6.
44. Allakhverdi Z, Comeau MR, Jessup HK, Yoon B-RP, Brewer A, Chartier S, Paquette N, Ziegler SF, Sarfati M, Delespesse G. Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. *J Exp Med* 2007;204:253–8.
45. McLeod JJA, Baker B, Ryan JJ. Mast cell production and response to IL-4 and IL-13. *Cytokine* 2015;75:57–61.
46. Bulfone-Paus S, Nilsson G, Draber P, Blank U, Levi-Schaffer F. Positive and Negative Signals in Mast Cell Activation. *Trends Immunol* 2017;38:657–667.
47. Kumar M, Duraisamy K, Chow B-K-C. Unlocking the Non-IgE-Mediated Pseudo-Allergic Reaction Puzzle with Mas-Related G-Protein Coupled Receptor Member X2 (MRGPRX2). *Cells* 2021;10:1033.
48. Ogasawara H, Noguchi M. Therapeutic potential of MRGPRX2 inhibitors on mast cells. *Cells* 2021;10:1–21.
49. Gaudenzio N, Sibilano R, Marichal T, Starkl P, Reber LL, Cenac N, McNeil BD, Dong X, Hernandez JD, Sagi-Eisenberg R, Hammel I, Roers A, Valitutti S, Tsai M, Espinosa E, Galli SJ. Different activation signals induce distinct mast cell degranulation strategies. *J Clin Invest* 2016;126:3981–3998.
50. Manorak W, Idahosa C, Gupta K, Roy S, Panettieri R, Ali H, Ali H. Upregulation of Mas-related G Protein coupled receptor X2 in asthmatic lung mast cells and its activation by the novel neuropeptide hemokinin-1. *Respir Res* 2018;19:1.
51. Krystel-Whittemore M, Dileepan KN, Wood JG. Mast Cell: A Multi-Functional Master Cell. *Front Immunol* 2015;6:620.
52. Carroll-Portillo A, Cannon JL, te Riet J, Holmes A, Kawakami Y, Kawakami T, Cambi A, Lidke DS. Mast cells and dendritic cells form synapses that facilitate antigen transfer for T cell activation. *J Cell Biol* 2015;210:851–864.
53. Idzko M, Sala A, Ferrari D, Panther E, Herouy Y, Dichmann S, Mockenhaupt M, Virgilio F Di, Girolomoni G, Norgauer J. Expression and function of histamine receptors in human monocyte-derived dendritic cells. *J Allergy Clin Immunol* 2002;109:839–846.
54. Dudeck J, Kotrba J, Immler R, Hoffmann A, Voss M, Alexaki VI, Morton L, Jahn SR, Katsoulis-Dimitriou K, Winzer S, Kollias G, Fischer T, Nedospasov SA, Dunay IR, Chavakis T, Müller AJ, Schraven B, Sperandio M, Dudeck A. Directional mast cell degranulation of tumor necrosis factor into blood vessels primes neutrophil extravasation. *Immunity* 2021;54:468-483.e5.
55. Dahdah A, Gautier G, Attout T, Fiore F, Lebourdais E, Msallam R, Daëron M, Monteiro RC, Benhamou M, Charles N, Davoust J, Blank U, Malissen B, Launay P. Mast cells aggravate sepsis by inhibiting peritoneal macrophage phagocytosis. *J Clin Invest* 2014;124:4577–4589.
56. Galdiero MR, Varricchi G, Seaf M, Marone G, Levi-Schaffer F, Marone G. Bidirectional mast cell-eosinophil interactions in inflammatory disorders and cancer. *Front Med* 2017;4:1–13.
57. Piliponsky AM, Gleich GJ, Nagler A, Bar I, Levi-Schaffer F. Non-IgE-dependent activation of human lung- and cord blood-derived mast cells is induced by eosinophil major basic protein and modulated by the membrane form of stem cell factor. *Blood* 2003;101:1898–1904.
58. Kortekaas Krohn I, Shikhagaie MM, Golebski K, Bernink JH, Breynaert C, Creyns B, Diamant Z, Fokkens WJ, Gevaert P, Hellings P, Hendriks RW, Klimek L, Mjösberg J, Morita H, Ogg GS, O'Mahony L, Schwarze J, Seys SF, Shamji MH, Bal SM. Emerging roles of innate lymphoid cells in inflammatory diseases: Clinical implications. *Allergy* 2018;73:837–850.
59. Barnig C, Cernadas M, Dutile S, Liu X, Perrella MA, Kazani S, Wechsler ME, Israel E, Levy BD. Lipoxin A4 regulates natural killer cell and type 2 innate lymphoid cell activation in asthma. *Sci Transl Med* 2013;5:174ra26.
60. Doherty TA, Khorram N, Lund S, Mehta AK, Croft M, Broide DH. Lung type 2 innate lymphoid cells express cysteinyl leukotriene receptor 1, which regulates TH2 cytokine production. *J Allergy Clin Immunol* 2013;132:205–13.
61. Xue L, Salimi M, Panse I, Mjösberg JM, McKenzie ANJ, Spits H, Klenerman P, Ogg G. Prostaglandin D2 activates group 2 innate lymphoid cells through chemoattractant receptor-homologous molecule expressed on TH2 cells. *J Allergy*

Clin Immunol 2014;133:1184-1194.e7.
62. Shimokawa C, Kanaya T, Hachisuka M, Ishiwata K, Hisaeda H, Kurashima Y, Kiyono H, Yoshimoto T, Kaisho T, Ohno H. Mast Cells Are Crucial for Induction of Group 2 Innate Lymphoid Cells and Clearance of Helminth Infections. *Immunity* 2017;46:863-874.e4.
63. Lefrançais E, Duval A, Mirey E, Roga S, Espinosa E, Cayrol C, Girard J-P. Central domain of IL-33 is cleaved by mast cell proteases for potent activation of group-2 innate lymphoid cells. *Proc Natl Acad Sci U S A* 2014;111:15502–7.
64. Hershko AY, Rivera J. Mast cell and T cell communication; amplification and control of adaptive immunity. *Immunol Lett* 2010;128:98–104.
65. Morita H, Arae K, Unno H, Miyauchi K, Toyama S, Nambu A, Oboki K, Ohno T, Motomura K, Matsuda A, Yamaguchi S, Narushima S, Kajiwara N, Iikura M, Suto H, McKenzie ANJ, Takahashi T, Karasuyama H, Okumura K, Azuma M, Moro K, Akdis CA, Galli SJ, Koyasu S, Kubo M, Sudo K, Saito H, Matsumoto K, Nakae S. An Interleukin-33-Mast Cell-Interleukin-2 Axis Suppresses Papain-Induced Allergic Inflammation by Promoting Regulatory T Cell Numbers. *Immunity* 2015;43:175–86.
66. Lai Y, Altemeier WA, Vandree J, Piliponsky AM, Johnson B, Appel CL, Frevert CW, Hyde DM, Ziegler SF, Smith DE, Henderson WR, Gelb MH, Hallstrand TS, Hallstrand TS. Increased density of intraepithelial mast cells in patients with exercise-induced bronchoconstriction regulated through epithelially derived thymic stromal lymphopoietin and IL-33. *J Allergy Clin Immunol* 2014;133:1448–55.
67. Cairns JA, Walls AF. Mast cell tryptase is a mitogen for epithelial cells. Stimulation of IL-8 production and intercellular adhesion molecule-1 expression. *J Immunol* 1996;156:275–83.
68. Begueret H, Berger P, Vernejoux JM, Dubuisson L, Marthan R, Tunon-De-Lara JM. Inflammation of bronchial smooth muscle in allergic asthma. *Thorax* 2007;62:8–15.
69. Kawabori S, Okuda M, Unno T, Nakamura A. Dynamics of mast cell degranulation in human allergic nasal epithelium after provocation with allergen. *Clin Allergy* 1985;15:509–15.
70. Altman MC, Lai Y, Nolin JD, Long S, Chen CC, Piliponsky AM, Altemeier WA, Larmore M, Frevert CW, Mulligan MS, Ziegler SF, Debley JS, Peters MC, Hallstrand TS. Airway epithelium–shifted mast cell infiltration regulates asthmatic inflammation via IL-33 signaling. *J Clin Invest* 2019;129:4979–4991.
71. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-Cell Infiltration of Airway Smooth Muscle in Asthma. *N Engl J Med* 2002;346:1699–1705.
72. Jiang J, Faiz A, Berg M, Carpaij OA, Vermeulen CJ, Brouwer S, Hesse L, Teichmann SA, ten Hacken NHT, Timens W, van den Berge M, Nawijn MC. Gene signatures from scRNA-seq accurately quantify mast cells in biopsies in asthma. *Clin Exp Allergy* 2020;50:1428–1431.
73. Salomonsson M, Malinovschi A, Kalm-Stephens P, Dahlin JS, Janson C, Alving K, Hallgren J. Circulating mast cell progenitors correlate with reduced lung function in allergic asthma. *Clin Exp Allergy* 2019;49:874–882.
74. Paivandy A, Pejler G. Novel Strategies to Target Mast Cells in Disease. *J Innate Immun* 2021;13:131–147.
75. Humbert M, Grant JA, Taborda-Barata L, Durham SR, Pfister R, Menz G, Barkans J, Ying S, Kay AB. High-affinity IgE receptor (FcepsilonRI)-bearing cells in bronchial biopsies from atopic and nonatopic asthma. *Am J Respir Crit Care Med* 1996;153:1931–1937.
76. Louis RE, Radermecker MF. Substance P-induced histamine release from human basophils, skin and lung fragments: effect of nedocromil sodium and theophylline. *Int Arch Allergy Appl Immunol* 1990;92:329–333.
77. Rönnberg E, Ravindran A, Mazzurana L, Gong Y, Säfholm J, Lorent J, Dethlefsen O, Orre AC, Al-Ameri M, Adner M, Dahlén SE, Dahlin JS, Mjösberg J, Nilsson G. Analysis of human lung mast cells by single cell RNA sequencing. *Front Immunol* 2023;14:1151754.
78. Siebenhaar F, Falcone FH, Tiligada E, Hammel I, Maurer M, Sagi-Eisenberg R, Levi-Schaffer F. The search for mast cell and basophil models - Are we getting closer to pathophysiological relevance? *Allergy Eur J Allergy Clin Immunol* 2015;70:1–5.
79. Laidlaw TM, Steinke JW, Tiñana AM, Feng C, Xing W, Lam BK, Paruchuri S, Boyce JA, Borish L. Characterization of a novel human mast cell line that responds to stem cell factor and expresses functional FcεRI. *J Allergy Clin Immunol* 2011;127:815-22.e1–5.
80. Saleh R, Wedeh G, Herrmann H, Bibi S, Cerny-Reiterer S, Sadovnik I, Blatt K, Hadzijusufovic E, Jeanningros S, Blanc C, Legarff-Tavernier M, Chapiro E, Nguyen-Khac F, Subra F, Bonnemye P, Dubreuil P, Desplat V, Merle-Béral H, Willmann M, Rülicke T, Valent P, Arock M. A new human mast cell line expressing a functional IgE receptor converts to tumorigenic growth by KIT D816V transfection. *Blood* 2014;124:111–120.
81. Andersen HB, Holm M, Hetland TE, Dahl C, Junker S, Schiøtz PO, Hoffmann HJ. Comparison of short term in vitro cultured human mast cells from different progenitors — Peripheral blood-derived progenitors generate highly mature and functional mast cells. *J Immunol Methods* 2008;336:166–174.
82. Shantanam S, MUELLER. An optimized protocol for the generation and functional analysis of human mast cells from CD34+ enriched cell populations. *Physiol Behav* 2018;176:139–148.
83. Lappalainen J, Lindstedt KA, Kovanen PT. A protocol for generating high numbers of mature and functional human mast cells from peripheral blood. *Clin Exp Allergy* 2007;37:1404–1414.
84. Holm M, Andersen HB, Hetland TE, Dahl C, Hoffmann HJ, Junker S, Schiøtz PO. Seven week culture of functional human mast cells from buffy coat preparations. *J Immunol Methods* 2008;336:213–221.
85. Valent P, Spanblochl E, Sperr WR, Sillaber C, Zsebo KM, Agis H, Strobl H, Geissler K, Bettelheim P, Lechner K. Induction of Differentiation of Human Mast Cells From Bone Marrow and Peripheral Blood Mononuclear Cells by Recombinant

Human Stem Cell Factor/kit-Ligand in Long-Term Culture. *Blood* 1992;80:2237–2245.
86. Derakhshan T, Bhowmick R, Ritchey JW, Gappa-Fahlenkamp H. Development of Human Mast Cells from Hematopoietic Stem Cells within a 3D Collagen Matrix: Effect of Stem Cell Media on Mast Cell Generation. *Stem Cells Int* 2018;2018:1–14.
87. Cop N, Decuyper I, Faber M, Sabato V, Bridts C, Hagendorens M, De Winter B, De Clerck L, Ebo D. Phenotypic and functional characterization of *in vitro* cultured human mast cells. *Cytom Part B Clin Cytom* 2017;92:348–354.
88. Wild CP. Complementing the Genome with an "Exposome": The Outstanding Challenge of Environmental Exposure Measurement in Molecular Epidemiology. *Cancer Epidemiol Prev Biomarkers* 2005;14:1847–1850.
89. Goossens J, Bullens DMA, Dupont LJ, Seys SF. Exposome mapping in chronic respiratory diseases: the added value of digital technology. *Curr Opin Allergy Clin Immunol* 2022;22:1–9.
90. De Greve G, Hellings PW, Fokkens WJ, Pugin B, Steelant B, Seys SF. Endotype-driven treatment in chronic upper airway diseases. *Clin Transl Allergy* 2017;7:22.
91. Muraro A, Lemanske RF, Hellings PW, Akdis CA, Bieber T, Casale TB, Jutel M, Ong PY, Poulsen LK, Schmid-Grendelmeier P, Simon H-U, Seys SF, Agache I. Precision medicine in patients with allergic diseases: Airway diseases and atopic dermatitis—PRACTALL document of the European Academy of Allergy and Clinical Immunology and the American Academy of Allergy, Asthma & Immunology. *J Allergy Clin Immunol* 2016;137:1347–1358.
92. Annesi-Maesano I, Maesano CN, Biagioni B, D'Amato G, Cecchi L. Call to action: Air pollution, asthma, and allergy in the exposome era. *J Allergy Clin Immunol* 2021;148:70–72.
93. Couto M, Kurowski M, Moreira A, Bullens DMA, Carlsen K-H, Delgado L, Kowalski ML, Seys SF. Mechanisms of exercise-induced bronchoconstriction in athletes: Current perspectives and future challenges. *Allergy* 2018;73:8–16.
94. Atchley TJ, Smith DM. Exercise-induced bronchoconstriction in elite or endurance athletes:: Pathogenesis and diagnostic considerations. *Ann Allergy, Asthma Immunol* 2020;125:1–8.
95. Teal S. Hallstrand, MD, MPHa, Jason S. Debley, MD, MPHb, Federico M. Farin, MDc, and William R. Henderson Jr M. Role of MUC5AC in the pathogenesis of exercise-induced bronchoconstriction Teal. *Bone* 2011;23:1–7.
96. Hox V, Vanoirbeek JA, Alpizar YA, Voedisch S, Callebaut I, Bobic S, Sharify A, De Vooght V, Van Gerven L, Devos F, Liston A, Voets T, Vennekens R, Bullens DMA, De Vries A, Hoet P, Braun A, Ceuppens JL, Talavera K, Nemery B, Hellings PW. Crucial Role of Transient Receptor Potential Ankyrin 1 and Mast Cells in Induction of Nonallergic Airway Hyperreactivity in Mice. *Am J Respir Crit Care Med* 2013;187:486–493.
97. Hallstrand TS, Moody MW, Aitken ML, Henderson WR. Airway immunopathology of asthma with exercise-induced bronchoconstriction. *J Allergy Clin Immunol* 2005;116:586–593.
98. Anderson SD, Kippelen P. Airway injury as a mechanism for exercise-induced bronchoconstriction in elite athletes. *J Allergy Clin Immunol* 2008;122:225–235.
99. Seys SF, Hox V, Van Gerven L, Dilissen E, Marijsse G, Peeters E, Dekimpe E, Kasran A, Aertgeerts S, Troosters T, Vanbelle V, Peers K, Ceuppens JL, Hellings PW, Dupont LJ, Bullens DM. Damage-associated molecular pattern and innate cytokine release in the airways of competitive swimmers. *Allergy* 2015;70:187–194.
100. Boulet L-P, O'Byrne PM. Asthma and exercise-induced bronchoconstriction in athletes. In: Drazen JM, editor. *N Engl J Med* 2015;372:641–8.
101. Jonckheere A-C, Seys S, Dilissen E, Schelpe A-S, Van der Eycken S, Corthout S, Verhalle T, Goossens J, Vanbelle V, Aertgeerts S, Troosters T, Peers K, Dupont L, Bullens D. Early-onset airway damage in early-career elite athletes: A risk factor for exercise-induced bronchoconstriction. *J Allergy Clin Immunol* 2019;144:1423-1425.e9.
102. Gomes EC, Stone V, Florida-James G. Impact of heat and pollution on oxidative stress and CC16 secretion after 8 km run. *Eur J Appl Physiol* 2011;111:2089–97.
103. Perry C, Pick M, Bdolach N, Hazan-Halevi I, Kay S, Berr I, Reches A, Harishanu Y, Grisaru D. Endurance exercise diverts the balance between Th17 cells and regulatory T cells. *PLoS One* 2013;8:e74722.
104. Bougault V, Turmel J, St-Laurent J, Bertrand M, Boulet L-P. Asthma, airway inflammation and epithelial damage in swimmers and cold-air athletes. *Eur Respir J* 2009;33:740–746.
105. Chimenti L, Morici G, Paternò A, Santagata R, Bonanno A, Profita M, Riccobono L, Bellia V, Bonsignore MR. Bronchial epithelial damage after a half-marathon in nonasthmatic amateur runners. *Am J Physiol Cell Mol Physiol* 2010;298:L857–L862.
106. Vaisberg M, Paulo S. Relation between TNF-alpha and exercise induced bronchoconstriction. 48.
107. Parsons JP, Baran CP, Phillips G, Jarjoura D, Kaeding C, Bringardner B, Wadley G, Marsh CB, Mastronarde JG. Airway Inflammation in Exercise-Induced Bronchospasm Occurring in Athletes Without Asthma. *J Asthma* 2008;45:363–367.
108. Hallstrand TS, Altemeier WA, Aitken ML, Henderson WR, Jr. Role of cells and mediators in exercise-induced bronchoconstriction. *Immunol Allergy Clin North Am* 2013;33:313–28, vii.
109. Goossens J, Jonckheere A-C, Dupont LJ, Bullens DMA. Air Pollution and the Airways: Lessons from a Century of Human Urbanization. *Atmosphere (Basel)* 2021;12:898.
110. Parsons JP, Hallstrand TS, Mastronarde JG, Kaminsky DA, Rundell KW, Hull JH, Storms WW, Weiler JM, Cheek FM, Wilson KC, Anderson SD. An official American thoracic society clinical practice guideline: Exercise-induced bronchoconstriction. *Am J Respir Crit Care Med* 2013;187:1016–1027.
111. Anderson SD, Kippelen P. Assessment and prevention of exercise-induced bronchoconstriction. *Br J Sports Med* 2012;46:391–6.
112. Weiler JM, Anderson SD, Randolph C, Bonini S, Craig TJ, Pearlman DS, Rundell KW, Silvers WS, Storms WW, Bernstein DI, Blessing-Moore J, Cox L, Khan DA, Lang DM, Nicklas RA, Oppenheimer J, Portnoy JM, Schuller DE, Spector SL, Tilles

SA, Wallace D, Henderson W, Schwartz L, Kaufman D, Nsouli T, Shieken L, Rosario N. Pathogenesis, prevalence, diagnosis, and management of exercise-induced bronchoconstriction: A practice parameter. *Ann Allergy, Asthma Immunol* 2010;105:S1–S47.
113. Del Giacco SR, Firinu D, Bjermer L, Carlsen K-H. Exercise and asthma: an overview. *Eur Clin Respir J* 2015;2:27984.
114. Carlsen KH, Anderson SD, Bjermer L, Bonini S, Brusasco V, Canonica W, Cummiskey J, Delgado L, Del Giacco SR, Drobnic F, Haahtela T, Larsson K, Palange P, Popov T, van Cauwenberge P. Exercise-induced asthma, respiratory and allergic disorders in elite athletes: epidemiology, mechanisms and diagnosis: Part I of the report from the Joint Task Force of the European Respiratory Society (ERS) and the European Academy of Allergy and Clinica. *Allergy* 2008;63:387–403.
115. Bonini M, Silvers W. Exercise-Induced Bronchoconstriction: Background, Prevalence, and Sport Considerations. *Immunol Allergy Clin North Am* 2018;38:205–214.
116. Bonini M, Gramiccioni C, Fioretti D, Ruckert B, Rinaldi M, Akdis C, Todaro A, Palange P, Carlsen K-HH, Pelliccia A, Rasi G, Bonini S, AIDA and the Italian Unit of the GA2LEN Olympic Study, AIDA. Asthma, allergy and the Olympics: a 12-year survey in elite athletes. *Curr Opin Allergy Clin Immunol* 2015;15:184–92.
117. Lomax M. Airway dysfunction in elite swimmers: prevalence, impact, and challenges. *Open Access J Sport Med* 2016;7:55.
118. Seys SF, Feyen L, Keirsbilck S, Adams E, Dupont LJ, Nemery B. An outbreak of swimming-pool related respiratory symptoms: An elusive source of trichloramine in a municipal indoor swimming pool. *Int J Hyg Environ Health* 2015;218:386–391.
119. Bougault V, Turmel J, Boulet L-P. Bronchial challenges and respiratory symptoms in elite swimmers and winter sport athletes: Airway hyperresponsiveness in asthma: its measurement and clinical significance. *Chest* 2010;138:31S-37S.
120. Hallstrand TS, Moody MW, Wurfel MM, Schwartz LB, Henderson WR, Aitken ML, Aitken ML. Inflammatory basis of exercise-induced bronchoconstriction. *Am J Respir Crit Care Med* 2005;172:679–86.
121. Clearie KL, Vaidyanathan S, Williamson PA, Goudie A, Short P, Schembri S, Lipworth BJ. Effects of chlorine and exercise on the unified airway in adolescent elite Scottish swimmers. *Allergy* 2010;65:269–73.
122. Rundell KW, Im J, Mayers LB, Wilber RL, Szmedra L, Schmitz HR. Self-reported symptoms and exercise-induced asthma in the elite athlete. *Med Sci Sports Exerc* 2001;33:208–213.
123. Parsons JP, Kaeding C, Phillips G, Jarjoura D, Wadley G, Mastronarde JG. Prevalence of Exercise-Induced Bronchospasm in a Cohort of Varsity College Athletes. *Med Sci Sport Exerc* 2007;39:1487–1492.
124. Dickinson J, McConnell A, Whyte G. Diagnosis of exercise-induced bronchoconstriction: eucapnic voluntary hyperpnoea challenges identify previously undiagnosed elite athletes with exercise-induced bronchoconstriction. *Br J Sport Med* 2011;45:1126–1131.
125. Bougault V, Loubaki L, Joubert P, Turmel J, Couture C, Laviolette M, Chakir J, Boulet L-P. Airway remodeling and inflammation in competitive swimmers training in indoor chlorinated swimming pools. *J Allergy Clin Immunol* 2012;129:351-358.e1.
126. Bougault V, Boulet LP. Airway dysfunction in swimmers. *Br J Sports Med* 2012;46:402–406.
127. Aguiar KB, Anzolin M, Zhang L. Global prevalence of exercise-induced bronchoconstriction in childhood: A meta-analysis. *Pediatr Pulmonol* 2018;53:412–425.
128. Price OJ, Hull JH, Backer V, Hostrup M, Ansley L. The impact of exercise-induced bronchoconstriction on athletic performance: A systematic review. *Sport Med* 2014;44:1749–1761.
129. Anderson SD, Argyros GJ, Magnussen H, Holzer K. Provocation by eucapnic voluntary hyperpnoea to identify exercise induced bronchoconstriction. *Br J Sports Med* 2001;35:344–7.
130. Van der Eycken S, Schelpe A, Marijsse G, Dilissen E, Troosters T, Vanbelle V, Aertgeerts S, Dupont LJ, Peers K, Bullens DM, Seys SF. Feasibility to apply eucapnic voluntary hyperventilation in young elite athletes. *Respir Med* 2016;111:91–93.
131. Hull JH, Ansley L, Price OJ, Dickinson JW, Bonini M. Eucapnic Voluntary Hyperpnea: Gold Standard for Diagnosing Exercise-Induced Bronchoconstriction in Athletes? *Sports Med* 2016;46:1083–93.
132. Anderson SD. 'Indirect' challenges from science to clinical practice. *Eur Clin Respir J* 2016;3:31096.
133. Shield S, Wangdolhlman A. Incidence of exercise-induced bronchospasm (EIB) in high school football players. *J Allergy Clin Immunol* 1991;87:166.
134. Marefati H, Hossaininasab M, Aghayari A, Boskabady MH, Mohseni M. Exercise induced bronchospasm in physically fit female students of Kerman University and their pulmonary function tests. *J Bodyw Mov Ther* 2012;16:338–343.
135. Bavarian B, Mehrkhani F, Ziaee V, Yousefi A, Nourian R. Sensitivity and specificity of self reported symptoms for exercise-induced bronchospasm diagnosis in children. *Iran J Pediatr* 2009;19:47–51.
136. Bougault V, Drouard F, Legall F, Dupont G, Wallaert B. Allergies and Exercise-Induced Bronchoconstriction in a Youth Academy and Reserve Professional Soccer Team. *Clin J Sport Med* 2017;27:450–456.
137. Feinstein RA, LaRussa J, Wang-Dohlman A, Bartolucci AA. Screening adolescent athletes for exercise-induced asthma. *Clin J Sport Med* 1996;6:119–23.
138. Hammerman SI, Becker JM, Rogers J, Quedenfeld TC, D'Alonzo GE. Asthma screening of high school athletes: Identifying the undiagnosed and poorly controlled. *Ann Allergy, Asthma Immunol* 2002;88:380–384.
139. Kukafka DS, Lang DM, Porter S, Rogers J, Ciccolella D, Polansky M, D'Alonzo GE. Exercise-induced Bronchospasm in High School Athletes via a Free Running Test: Incidence and Epidemiology. *Chest* 1998;114:1613–1622.
140. Mansournia MA, Jamali M, Mansournia N, Yunesian M, Moghadam KG. Exercise-induced bronchospasm among

students of Tehran University of Medical Sciences in 2004. *Allergy Asthma Proc* 2007;28:348–352.
141. Molphy J, Dickinson J, Hu J, Chester N, Whyte G. Prevalence of bronchoconstriction induced by eucapnic voluntary hyperpnoea in recreationally active individuals. *J Asthma* 2014;51:44–50.
142. Sallaoui R, Zendah I, Ghedira H, Belhaouz M, Ghrairi M, Amri M. Exercise-induced bronchoconstriction in Tunisian elite athletes is underdiagnosed. *Open access J Sport Med* 2011;2:41–6.
143. Thole RT, Sallis RE, Rubin AL, Smith GN. Exercise-induced bronchospasm prevalence in collegiate cross-country runners. *Med Sci Sports Exerc* 2001;33:1641–1646.
144. Vakali S, Vogiatzis I, Florou A, Giavi S, Zakynthinos S, Papadopoulos NG, Gratziou C. Exercice-induced bronchoconstriction among athletes: Assessment of bronchial provocation tests. *Respir Physiol Neurobiol* 2017;235:34–39.
145. Romberg K, Tufvesson E, Bjermer L. Extended diagnostic criteria used for indirect challenge testing in elite asthmatic swimmers. *Respir Med* 2012;106:15–24.
146. Burnett DM, Vardiman JP, Deckert JA, Ward JL, Sharpe MR. Perception of Exercise-Induced Bronchoconstriction in College Athletes. *Respir Care* 2016;61:897–901.
147. Hallstrand TS, Curtis JR, Koepsell TD, Martin DP, Schoene RB, Sullivan SD, Yorioka GN, Aitken ML. Effectiveness of screening examinations to detect unrecognized exercise-induced bronchoconstriction. *J Pediatr* 2002;141:343–349.
148. Parsons JP, Cosmar D, Phillips G, Kaeding C, Best TM, Mastronarde JG. Screening for exercise-induced bronchoconstriction in college athletes. *J Asthma* 2012;49:153–7.
149. Rundell KW, Wilber RL, Szmedra L, Jenkinson DM, Mayers LB, Im J. Exercise-induced asthma screening of elite athletes: field versus laboratory exercise challenge. *Med Sci Sports Exerc* 2000;32:309–16.
150. Rupp NT, Brudno DS, Guill MF. The value of screening for risk of exercise-induced asthma in high school athletes. *Ann Allergy* 1993;70:339–42.
151. Burnett DM, Burns S, Merritt S, Wick J, Sharpe M. Prevalence of Exercise-Induced Bronchoconstriction Measured by Standardized Testing in Healthy College Athletes. *Respir Care* 2016;61:571–576.
152. Weiler JM, Brannan JD, Randolph CC, Hallstrand TS, Parsons J, Silvers W, Storms W, Zeiger J, Bernstein DI, Blessing-Moore J, Greenhawt M, Khan D, Lang D, Nicklas RA, Oppenheimer J, Portnoy JM, Schuller DE, Tilles SA, Wallace D. Exercise-induced bronchoconstriction update—2016. *J Allergy Clin Immunol* 2016;138:1292-1295.e36.
153. Helenius IJ, Tikkanen HO, Sarna S, Haahtela T. Asthma and increased bronchial responsiveness in elite athletes: Atopy and sport event as risk factors. *J Allergy Clin Immunol* 1998;101:646–652.
154. Helenius IJ, Tikkanen HO, Haahtela T. Occurrence of exercise induced bronchospasm in elite runners: dependence on atopy and exposure to cold air and pollen. *Br J Sports Med* 1998;32:125–9.
155. Bonini M, Braido F, Baiardini I, Del Giacco S, Gramiccioni C, Manara M, Tagliapietra G, Scardigno A, Sargentini V, Brozzi M, Rasi G, Bonini S. AQUA: Allergy Questionnaire for Athletes. Development and validation. *Med Sci Sports Exerc* 2009;41:1034–41.
156. Jonckheere A-C, Seys SF, Dilissen E, Marijsse G, Schelpe A-S, Van der Eycken S, Verhalle T, Vanbelle V, Aertgeerts S, Troosters T, Peers K, Dupont LJ, Bullens DMA. AQUA © Questionnaire as prediction tool for atopy in young elite athletes. *Pediatr Allergy Immunol* 2018;29:648–650.
157. Fasola S, Bellafiore M, Baiardini I, Bonini M, Del Giacco S, Ferrante G, La Grutta S. Feasibility of the Allergy Questionnaire for Athletes (AQUA©) in pediatric age. *Pediatr Allergy Immunol* 2019;30:242–245.
158. Fasola S, Ferrante G, Malizia V, Bommarito N, Stefano |, Giacco D, Bonini M, Baiardini I, Bellafiore M, Stefania |, Grutta L. Validity and repeatability of the Pediatric Allergy Questionnaire for Athletes (AQUAped) for the screening of atopy. *Pediatr Allergy Immunol* 2021;32:437–444.
159. Allen H, Hull JH, Backhouse SH, De Carné T, Dimitriou L, Price OJ. The Allergy Questionnaire for Athletes provides value in ruling-out exercise-induced bronchoconstriction. *Allergy* 2019;74:1794–1796.
160. Fitch KD. An overview of asthma and airway hyper-responsiveness in Olympic athletes. *Br J Sports Med* 2012;46:413–6.
161. Steelant B, Hox V, Hellings PW, Bullens DM, Seys SF. Exercise and Sinonasal Disease. *Immunol Allergy Clin North Am* 2018;38:259–269.
162. Kockentiet B, Adams BB. Contact dermatitis in athletes. *J Am Acad Dermatol* 2007;56:1048–55.
163. Katelaris CH, Carrozzi FM, Burke T V, Byth K. A springtime olympics demands special consideration for allergic athletes. *J Allergy Clin Immunol* 2000;106:260–6.
164. Zwick H, Popp W, Budik G, Wanke T, Rauscher H. Increased sensitization to aeroallergens in competitive swimmers. *Lung* 1990;168:111–115.
165. Teixeira RN, Leite G dos S, Bonini M, Gorjão R, Agondi RC, Kokron CM, Carvalho CRF. Atopy in Elite Endurance Athletes. *Clin J Sport Med* 2018;28:268–271.
166. Pepys J. Natural history of "atopy". *J Allergy Clin Immunol* 1986;78:959–61.
167. Sallaoui R, Chamari K, Mossa A, Tabka Z, Chtara M, Feki Y, Amri M. Exercise-induced bronchoconstriction and atopy in Tunisian athletes. *BMC Pulm Med* 2009;9:8.
168. Menzies-Gow A, Mansur AH, Brightling CE. Clinical utility of fractional exhaled nitric oxide in severe asthma management. *Eur Respir J* 2020;55:1901633.
169. Dweik RA, Boggs PB, Erzurum SC, Irvin CG, Leigh MW, Lundberg JO, Olin A-C, Plummer AL, Taylor DR. An Official ATS Clinical Practice Guideline: Interpretation of Exhaled Nitric Oxide Levels (F <scp>e</scp> $_{NO}$) for Clinical Applications. *Am J Respir Crit Care Med* 2011;184:602–615.

170. Karrasch S, Ernst K, Behr J, Heinrich J, Huber RM, Nowak D, Wichmann HE, Baumeister SE, Meisinger C, Ladwig KH, Holle R, Jörres RA, Schulz H. Exhaled nitric oxide and influencing factors in a random population sample. *Respir Med* 2011;105:713–718.
171. Bonini M, Messina J, Petrarulo S, Piamonti D, Di Paolo M, Brunori M, Palange P, Usmani O. Baseline FeNO is an independent predictive marker of Exercise-Induced Bronchoconstriction (EIB). *Clin Respir Physiol Exerc Funct imaging* European Respiratory Society; 2018. p. PA2419.
172. Allen H, Hull J, Backhouse S, Dickinson J, De Carné T, Dimitriou L, Price O. The role of fractional exhaled nitric oxide in the assessment of athletes reporting exertional dyspnoea. *Allergy* 2019;74:.
173. Henrik Johansson, Christer Janson, Katarina Norlander, Kjell Alving, Leif Nordang, Lennart Nordvall, Andrei Malinovschi ME. Elevated exhaled NO is a predictor of exercise-induced bronchoconstriction in adolescents. *Eur Respir J* 2014;44:1005.
174. Voutilainen M, Malmberg LP, Vasankari T, Haahtela T. Exhaled nitric oxide indicates poorly athlete's asthma. *Clin Respir J* 2013;7:347–353.
175. Millward D, Paul S, Brown M, Porter D, Stilson M, Cohen R, Olvey E, Hagan J. The diagnosis of asthma and exercise-induced bronchospasm in division I athletes. *Clin J Sport Med* 2009;19:482–6.
176. Pedersen L, Lund TK, Barnes PJ, Kharitonov SA, Backer V. Airway responsiveness and inflammation in adolescent elite swimmers. *J Allergy Clin Immunol* 2008;122:322-327.e1.
177. Ersson K, Johansson H, Mallmin E, Malinovschi A. Exercise-induced bronchoconstriction (EIB) is not associated with exhaled nitric oxide (FeNO) in adolescent athletes. *Paediatr asthma allergy* European Respiratory Society; 2019. p. PA4508.doi:10.1183/13993003.congress-2019.PA4508.
178. Parsons JP, Cosmar D, Phillips G, Kaeding C, Best T, Mastronarde JG. Lack of efficacy in screening for EIB in college athletes. 2011;doi:10.1164/ajrccm-conference.2011.183.1_MeetingAbstracts.A4419.
179. Scollo M, Zanconato S, Ongaro R, Zaramella C, Zacchello F, Baraldi E. Exhaled nitric oxide and exercise-induced bronchoconstriction in asthmatic children. *Am J Respir Crit Care Med* 2000;161:1047–1050.
180. Kasmay Ö, Yldrm A, Ünal M, Kaçar Ö, Bilsel S, Kurtel H. The involvement of nitric oxide and endothelin-1 in exercise-induced bronchospasm in young soccer players. *Clin J Sport Med* 2011;21:237–242.
181. Seys SF, Lokwani R, Simpson JL, Bullens DMA. New insights in neutrophilic asthma. *Curr Opin Pulm Med* 2019;25:113–120.
182. Seys SF, Grabowski M, Adriaensen W, Decraene A, Dilissen E, Vanoirbeek JA, Dupont LJ, Ceuppens JL, Bullens DMA. Sputum cytokine mapping reveals an 'IL-5, IL-17A, IL-25-high' pattern associated with poorly controlled asthma. *Clin Exp Allergy* 2013;43:1009–1017.
183. Seys SF, Scheers H, Van den Brande P, Marijsse G, Dilissen E, Van Den Bergh A, Goeminne PC, Hellings PW, Ceuppens JL, Dupont LJ, Bullens DMA. Cluster analysis of sputum cytokine-high profiles reveals diversity in T(h)2-high asthma patients. *Respir Res* 2017;18:39.
184. Decaesteker T, Seys S, Hox V, Dilissen E, Marijsse G, Manhaeghe L, Hellings P, Vanoirbeek J, Bullens D, Dupont L. Serum and sputum calprotectin, a reflection of neutrophilic airway inflammation in asthmatics after high-altitude exposure. *Clin Exp Allergy* 2017;47:1675–1677.
185. Nielsen C, Lang RS. PRINCIPLES OF SCREENING. *Med Clin North Am* 1999;83:1323–1337.
186. Carlsen KH, Anderson SD, Bjermer L, Bonini S, Brusasco V, Canonica W, Cummiskey J, Delgado L, Del Giacco SR, Drobnic F, Haahtela T, Larsson K, Palange P, Popov T, Van Cauwenberge P. Exercise-induced asthma, respiratory and allergic disorders in elite athletes: epidemiology, mechanisms and diagnosis: Part I of the report from the Joint Task Force of the European Respiratory Society (ERS) and the European Academy of Allergy and Clinica. *Allergy* 2008;63:387–403.
187. Burns J, Mason C, Mueller N, Ohlander J, Zock J-P, Drobnic F, Wolfarth B, Heinrich J, Omenaas E, Stensrud T, Nowak D, Radon K. Asthma prevalence in Olympic summer athletes and the general population: An analysis of three European countries. *Respir Med* 2015;109:813–820.
188. Bonini M, Gramiccioni C, Fioretti D, Ruckert B, Rinaldi M, Akdis C, Todaro A, Palange P, Carlsen KH, Pelliccia A, Rasi G, Bonini S, AIDA. Asthma, allergy and the olympics: A 12-year survey in elite athletes. *Curr Opin Allergy Clin Immunol* 2015;15:184–192.
189. Price OJ, Sewry N, Schwellnus M, Backer V, Reier-Nilsen T, Bougault V, Pedersen L, Chenuel B, Larsson K, Hull JH. Prevalence of lower airway dysfunction in athletes: a systematic review and meta-analysis by a subgroup of the IOC consensus group on 'acute respiratory illness in the athlete.' *Br J Sports Med* 2022;56:213–222.
190. Eigenmann PA, Atanaskovic-Markovic M, O'B Hourihane J, Lack G, Lau S, Matricardi PM, Muraro A, Namazova Baranova L, Nieto A, Papadopoulos NG, Réthy LA, Roberts G, Rudzeviciene O, Wahn U, Wickman M, Høst A. Testing children for allergies: Why, how, who and when: An updated statement of the European Academy of Allergy and Clinical Immunology (EAACI) Section on Pediatrics and the EAACI-Clemens von Pirquet Foundation. *Pediatr Allergy Immunol* 2013;24:195–209.
191. Gagliardo R, La Grutta S, Chanez P, Profita M, Paternò A, Cibella F, Bousquet J, Viegi G, Gjomarkaj M. Non-invasive markers of airway inflammation and remodeling in childhood asthma. *Pediatr Allergy Immunol* 2009;20:780–790.
192. Rasmussen SM, Halvard Hansen ES, Stensrud T, Radon K, Wolfarth B, Kurowski M, Bousquet J, Bonini S, Bonini M, Delgado L, Moreira A, Drobnic F, Papadopoulos NG, Vakali S, Gratziou C, Malmberg LP, Haahtela T, Backer V. Asthma endotypes in elite athletes: A cross-sectional study of European athletes participating in the Olympic Games. *Allergy Eur J Allergy Clin Immunol* 2022;77:2250–2253.

193. Martins C, Silva D, Severo M, Rufo J, Paciência I, Madureira J, Padrão P, Moreira P, Luís Delgado |, Fernandes EO, Barros H, Malmberg P, Moreira | André. Spirometry-adjusted fraction of exhaled nitric oxide increases accuracy for assessment of asthma control in children. *Pediatr Allergy Immunol* 2017;28:754–762.
194. Linhares D, Jacinto T, Pereira AM, Fonseca JA. Effects of atopy and rhinitis on exhaled nitric oxide values - a systematic review. *Clin Transl Allergy* 2011;1:1–7.
195. For a healthy Belgium. Overview - Non-Communicable Diseases. 2022;at <https://www.healthybelgium.be/en/health-status/non-communicable-diseases/overview>.
196. Yao TC, Ou LS, Lee WI, Yeh KW, Chen LC, Huang JL. Exhaled nitric oxide discriminates children with and without allergic sensitization in a population-based study. *Clin Exp Allergy* 2011;41:556–564.
197. Romero KM, Robinson CL, Baumann LM, Gilman RH, Hamilton RG, Hansel NN, Checkley W. Role of exhaled nitric oxide as a predictor of atopy. *Respir Res* 2013;14:1–8.
198. Latimer-Cheung AE, Toll BA, Salovey P. Promoting increased physical activity and reduced inactivity. *Lancet* 2013;381:114.
199. Moreira A, Bonini M, Pawankar R, Anderson SD, Carlsen K-H, Randolph C, Silvers W, Storms W, Weiler JM, Bonini S. A World Allergy Organization international survey on physical activity as a treatment option for asthma and allergies. *World Allergy Organ J* 2014;7:34.
200. Goossens J, Jonckheere A-C, Seys SF, Dilissen E, Decaesteker T, Goossens C, Peers K, Vanbelle V, Stappers J, Aertgeerts S, De Wilde B, Leus J, Verelst S, Raes M, Dupont L, Bullens DM. Activation of epithelial and inflammatory pathways in adolescent elite athletes exposed to intense exercise and air pollution. *Thorax* 2023;doi:10.1136/thorax-2022-219651.
201. Fitch KD, Sue-Chu M, Anderson SD, Boulet L-P, Hancox RJ, McKenzie DC, Backer V, Rundell KW, Alonso JM, Kippelen P, Cummiskey JM, Garnier A, Ljungqvist A. Asthma and the elite athlete: Summary of the International Olympic Committee's Consensus Conference, Lausanne, Switzerland, January 22-24, 2008. *J Allergy Clin Immunol* 2008;122:254-260.e7.
202. Price OJ, Walsted ES, Bonini M, Brannan JD, Bougault V, Carlsen KH, Couto M, Kippelen P, Moreira A, Pite H, Rukhadze M, Hull JH. Diagnosis and management of allergy and respiratory disorders in sport: An EAACI task force position paper. *Allergy Eur J Allergy Clin Immunol* 2022;77:2909–2923.
203. Levai IK, Hull JH, Loosemore M, Greenwell J, Whyte G, Dickinson JW. Environmental influence on the prevalence and pattern of airway dysfunction in elite athletes. *Respirology* 2016;21:1391–1396.
204. Schwellnus M, Adami PE, Bougault V, Budgett R, Clemm HH, Derman W, Erdener U, Fitch K, Hull JH, McIntosh C, Meyer T, Pedersen L, Pyne DB, Reier-Nilsen T, Schobersberger W, Schumacher YO, Sewry N, Soligard T, Valtonen M, Webborn N, Engebretsen L. International Olympic Committee (IOC) consensus statement on acute respiratory illness in athletes part 2: non-infective acute respiratory illness. *Br J Sports Med* 2022;56:1089–1103.
205. Goossens J, Decaesteker T, Jonckheere A-C, Seys S, Verelst S, Dupont L, Bullens DMA. How to detect young athletes at risk of exercise-induced bronchoconstriction? *Paediatr Respir Rev* 2022;44:40–46.
206. American Thoracic Society, European Respiratory Society. ATS/ERS Recommendations for Standardized Procedures for the Online and Offline Measurement of Exhaled Lower Respiratory Nitric Oxide and Nasal Nitric Oxide, 2005. *Am J Respir Crit Care Med* 2005;171:912–930.
207. Graham BL, Steenbruggen I, Miller MR, Barjaktarevic IZ, Cooper BG, Hall GL, Hallstrand TS, Kaminsky DA, McCarthy K, McCormack MC, Oropez CE, Rosenfeld M, Stanojevic S, Swanney MP, Thompson BR. Standardization of Spirometry 2019 Update. An Official American Thoracic Society and European Respiratory Society Technical Statement. *Am J Respir Crit Care Med* 2019;200:e70–e88.
208. Cooper BG, Stocks J, Hall GL, Culver B, Steenbruggen I, Carter KW, Thompson BR, Graham BL, Miller MR, Ruppel G, Henderson J, Vaz Fragoso CA, Stanojevic S. The Global Lung Function Initiative (GLI) Network: bringing the world's respiratory reference values together. *Breathe* 2017;13:e56–e64.
209. Heinzerling L, Mari A, Bergmann K-C, Bresciani M, Burbach G, Darsow U, Durham S, Fokkens W, Gjomarkaj M, Haahtela T, Bom AT, Wöhrl S, Maibach H, Lockey R. The skin prick test – European standards. *Clin Transl Allergy* 2013;3:3.
210. Schatz M, Sorkness CA, Li JT, Marcus P, Murray JJ, Nathan RA, Kosinski M, Pendergraft TB, Jhingran P. Asthma Control Test: Reliability, validity, and responsiveness in patients not previously followed by asthma specialists. *J Allergy Clin Immunol* 2006;117:549–556.
211. Juniper EF, Svensson K, Mörk AC, Ståhl E. Measurement properties and interpretation of three shortened versions of the asthma control questionnaire. *Respir Med* 2005;99:553–558.
212. Couto M, Stang J, Horta L, Stensrud T, Severo M, Mowinckel P, Silva D, Delgado L, Moreira A, Carlsen K-H. Two distinct phenotypes of asthma in elite athletes identified by latent class analysis. *J Asthma* 2015;52:897–904.
213. Couto M, Bernard A, Delgado L, Drobnic F, Kurowski M, Moreira A, Rodrigues-Alves R, Rukhadze M, Seys S, Wiszniewska M, Quirce S. Health effects of exposure to chlorination by-products in swimming pools. *Allergy* 2021;76:3257–3275.
214. Thomas S, Wolfarth B, Wittmer C, Nowak D, Radon K. IMMUNOLOGY Self-reported asthma and allergies in top athletes compared to the general population - results of the German part of the GA 2 LEN-Olympic study 2008. *Allergy, Asthma Clin Immunol* 2010;6:31.
215. Ehrlich RI, Du Toit D, Jordaan E, Zwarenstein M, Potter P, Volmink JA, Weinberg E. Risk factors for childhood asthma and wheezing. Importance of maternal and household smoking. *Am J Respir Crit Care Med* 1996;154:681–688.

216. Anderson SD, Kippelen P. Exercise-induced bronchoconstriction: Pathogenesis. *Curr Allergy Asthma Rep* 2005;5:116–122.
217. Bonini M, Lapucci G, Petrelli G, Todaro A, Pamich T, Rasi G, Bonini S. Predictive value of allergy and pulmonary function tests for the diagnosis of asthma in elite athletes. *Allergy* 2007;62:1166–1170.
218. Rouhos A, Ekroos H, Karjalainen S, Sarna S, Sovijärvi ARA. Exhaled nitric oxide and exercise-induced bronchoconstriction in young male conscripts: association only in atopics. *Allergy* 2005;60:1493–1498.
219. Bonini M, Usmani OS. Let research leave you breathless, not physical exercise! *ERJ Open Res* 2018;4:00010–02018.
220. Goossens J, Vandekerckhove J, Jonckheere A-C, Dilissen E, Seys SF, Vanbelle V, Aertgeerts S, Stappers J, Peers K, Raes M, Verelst S, Leus J, Dupont LJ, Bullens DMA. Can AQUA© questionnaire and FeNO predict atopy in early-career athletes? *Pediatr Allergy Immunol* 2023;34:e13936.
221. Goh J, Hofmann P, Aw NH, Tan PL, Tschakert G, Mueller A, Wong SC, Tan F, Seo L, Gan H. Concurrent high-intensity aerobic and resistance exercise modulates systemic HSP70) and inflammatory biomarkers in healthy young men : a pilot study. *Transl Med Commun* 2020;5:4.
222. Frey A, Lunding LP, Ehlers JC, Weckmann M, Zissler UM, Wegmann M. More Than Just a Barrier: The Immune Functions of the Airway Epithelium in Asthma Pathogenesis. *Front Immunol* 2020;11:761.
223. Parsons JP, Mastronarde JG. Exercise-Induced Bronchoconstriction in Athletes. *Chest* 2005;128:3966–3974.
224. Thurston GD, Kipen H, Annesi-Maesano I, Balmes J, Brook RD, Cromar K, De Matteis S, Forastiere F, Forsberg B, Frampton MW, Grigg J, Heederik D, Kelly FJ, Kuenzli N, Laumbach R, Peters A, Rajagopalan ST, Rich D, Ritz B, Samet JM, Sandstrom T, Sigsgaard T, Sunyer J, Brunekreef B. A joint ERS/ATS policy statement: what constitutes an adverse health effect of air pollution? An analytical framework. *Eur Respir J* 2017;49:1600419.
225. Rundell KW. Effect of air pollution on athlete health and performance. *Br J Sports Med* 2012;46:407–412.
226. Zhang J, Zhu T, Kipen H, Wang G, Huang W, Rich D, Zhu P, Wang Y, Lu S-E, Ohman-Strickland P, Diehl S, Hu M, Tong J, Gong J, Thomas D, HEI Health Review Committee. Cardiorespiratory biomarker responses in healthy young adults to drastic air quality changes surrounding the 2008 Beijing Olympics. *Res Rep Health Eff Inst* 2013;5–174.
227. Bruce XRM. The control of ventilation during exercise: a lesson in critical thinking. *Adv Physiol Educ* 2017;41:539–547.
228. Kippelen P, Fitch KD, Anderson SD, Bougault V, Boulet LP, Rundell KW, Sue-Chu M, McKenzie DC. Respiratory health of elite athletes - preventing airway injury: a critical review. *Br J Sports Med* 2012;46:471–476.
229. Guarnieri M, Balmes JR. Outdoor air pollution and asthma. *Lancet* 2014;383:1581.
230. Daigle CC, Chalupa DC, Gibb FR, Morrow PE, Oberdörster G, Utell MJ, Frampton MW. Ultrafine particle deposition in humans during rest and exercise. *Inhal Toxicol* 2003;15:539–52.
231. McDonnell WF, Horstman DH, Hazucha MJ, Seal E, Haak ED, Salaam SA, House DE. Pulmonary effects of ozone exposure during exercise: dose-response characteristics. *J Appl Physiol* 1983;54:1345–52.
232. Weiszhar Z, Horvath I. Induced sputum analysis: step by step. *Breathe* 2013;9:300–306.
233. Metzker ML. Sequencing technologies — the next generation. *Nat Rev Genet* 2009;doi:10.1038/nrg2626.
234. Seys SF, Daenen M, Dilissen E, Van Thienen R, Bullens DMA, Hespel P, Dupont LJ. Effects of high altitude and cold air exposure on airway inflammation in patients with asthma. *Thorax* 2013;68:906–13.
235. Truyen E, Coteur L, Dilissen E, Overbergh L, Dupont LJ, Ceuppens JL, Bullens DMA. Evaluation of airway inflammation by quantitative Th1/Th2 cytokine mRNA measurement in sputum of asthma patients. *Thorax* 2006;61:202–8.
236. Bullens DMA, Truyen E, Coteur L, Dilissen E, Hellings PW, Dupont LJ, Ceuppens JL. IL-17 mRNA in sputum of asthmatic patients: Linking T cell driven inflammation and granulocytic influx? *Respir Res* 2006;7:135.
237. Bai Y, Bové H, Nawrot TS, Nemery B. Carbon load in airway macrophages as a biomarker of exposure to particulate air pollution; A longitudinal study of an international Panel. *Part Fibre Toxicol* 2018;15:1–10.
238. Air Quality Guidelines Global Update 2005. 2006;at <www.euro.who.int>.
239. Nieman DC, Wentz LM. The compelling link between physical activity and the body's defense system. *J Sport Heal Sci* 2019;8:201.
240. Suzuki K. Cytokine Response to Exercise and Its Modulation. *Antioxidants 2018, Vol 7, Page 17* 2018;7:17.
241. Santos J de MB Dos, Bachi ALL, Luna Junior LA, Foster R, Sierra APR, Benetti M, Araújo JR, Ghorayeb N, Kiss MAPD, Vieira RP, Bullens DMA, Vaisberg M. The Relationship of IL-8 and IL-10 Myokines and Performance in Male Marathon Runners Presenting Exercise-Induced Bronchoconstriction. *Int J Environ Res Public Health* 2020;17:1–15.
242. Bai Y, Brugha RE, Jacobs L, Grigg J, Nawrot TS, Nemery B. Carbon loading in airway macrophages as a biomarker for individual exposure to particulate matter air pollution - A critical review. *Environ Int* 2015;74:32–41.
243. Belli AJ, Bose S, Aggarwal N, DaSilva C, Thapa S, Grammer L, Paulin LM, Hansel NN. Indoor particulate matter exposure is associated with increased black carbon content in airway macrophages of former smokers with COPD. *Environ Res* 2016;150:398.
244. Fullerton DG, Jere K, Jambo K, Kulkarni NS, Zijlstra EE, Grigg J, French N, Molyneux ME, Gordon SB. Domestic smoke exposure is associated with alveolar macrophage particulate load. *Trop Med Int Health* 2009;14:349–354.
245. Jacobs L, Emmerechts J, Mathieu C, Hoylaerts MF, Fierens F, Hoet PH, Nemery B, Nawrot TS. Air Pollution–Related Prothrombotic Changes in Persons with Diabetes. *Environ Health Perspect* 2010;118:191.
246. Steinvil A, Fireman E, Kordova-Biezuner L, Cohen M, Shapira I, Berliner S, Rogowski O. Environmental Air Pollution Has Decremental Effects on Pulmonary Function Test Parameters Up to One Week After Exposure. *Am J Med Sci* 2009;338:273–279.
247. Chang YK, Wu CC, Lee LT, Lin RS, Yu YH, Chen YC. The short-term effects of air pollution on adolescent lung function

in Taiwan. *Chemosphere* 2012;87:26–30.
248. Rizzo JÂ, Rodrigues Filho E de A, Gonçalves AV, Albuquerque LCBDB, Albuquerque EM de, Albuquerque CG de, Almeida AHS de, Peixoto DM, Correia Junior MA de V. Reproducibility of eucapnic voluntary hyperpnoea for exercise-induced bronchoconstriction diagnosis in asthmatic children and adolescents. In: Kalayci Ö, editor. *Pediatr Allergy Immunol* 2021;32:1700–1708.
249. Price OJ, Ansley L, Hull JH. Diagnosing Exercise-Induced Bronchoconstriction With Eucapnic Voluntary Hyperpnea: Is One Test Enough? *J Allergy Clin Immunol Pract* 2015;3:243–249.
250. Bonsignore MR, Morici G, Vignola AM, Riccobono L, Bonanno A, Profita M, Abate P, Scichilone N, Amato G, Bellia V, Bonsignore G. Increased airway inflammatory cells in endurance athletes: what do they mean? *Clin Exp Allergy* 2003;33:14–21.
251. Belda J, Ricart S, Casan P, Giner J, Bellido-Casado J, Torrejon M, Margarit G, Drobnic F. Airway inflammation in the elite athlete and type of sport. *Br J Sports Med* 2008;42:244–248.
252. Vijayaraghava A, K R. Alteration of Interferon Gamma (IFN-γ) in Human Plasma with Graded Physical Activity. *J Clin Diagn Res* 2014;8:BC05-7.
253. Gleeson M, McFarlin B, Flynn M. Exercise and Toll-like receptors. *Exerc Immunol Rev* 2006;12:34–53.
254. Collao N, Rada I, Francaux M, Deldicque L, Zbinden-Foncea H. Anti-Inflammatory Effect of Exercise Mediated by Toll-Like Receptor Regulation in Innate Immune Cells - A Review. *Int Rev Immunol* 2020;39:39–52.
255. Ho AW, Gaffen SL. IL-17RC: A partner in IL-17 signaling and beyond. *Semin Immunopathol* 2010;32:33.
256. Xie F, Xiao P, Chen D, Xu L, Zhang B. miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. *Plant Mol Biol* 2012;80:75–84.
257. Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* 2001;25:386–401.
258. Aronesty E. Command-line tools for processing biological sequencing data. *ea-utils* 2011;at <https://github.com/ExpressionAnalysis/ea-utils>.
259. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 2009;25:2078.
260. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15:550.
261. Liu T, Zhang L, Joo D, Sun S-C. NF-κB signaling in inflammation. *Signal Transduct Target Ther* 2017;2:17023.
262. Hadžović-Džuvo A, Valjevac A, Lepara O, Pjanić S, Hadžimuratović A, Mekić A. Oxidative stress status in elite athletes engaged in different sport disciplines. *Bosn J Basic Med Sci* 2014;14:56.
263. Gauthier M, Ray A, Wenzel SE. Evolving concepts of asthma. *Am J Respir Crit Care Med* 2015;192:660–668.
264. Heijink IH, Kuchibhotla VNS, Roffel MP, Maes T, Knight DA, Sayers I, Nawijn MC. Epithelial cell dysfunction, a major driver of asthma development. *Allergy Eur J Allergy Clin Immunol* 2020;75:1898–1913.
265. Strzelak A, Ratajczak A, Adamiec A, Feleszko W. Tobacco Smoke Induces and Alters Immune Responses in the Lung Triggering Inflammation, Allergy, Asthma and Other Lung Diseases: A Mechanistic Review. *Int J Environ Res Public Health* 2018;15:1033.
266. Lummus ZL, Wisnewski A V., Bernstein DI. Pathogenesis and Disease Mechanisms of Occupational Asthma. *Immunol Allergy Clin North Am* 2011;31:699–716.
267. Porsbjerg C, Menzies-Gow A. Co-morbidities in severe asthma: Clinical impact and management. *Respirology* 2017;22:651–661.
268. Boulet LP. Influence of comorbid conditions on asthma. *Eur Respir J* 2009;33:897–906.
269. Pizzichini E, Pizzichini MMM, Efthimiadis A, Hargreave FE, Dolovich J. Measurement of inflammatory indices in induced sputum: effects of selection of sputum to minimize salivary contamination. *Eur Respir J* 1996;9:1174–1180.
270. Vizcaya D, Mirabelli MC, Orriols R, Antó JM, Barreiro E, Burgos F, Arjona L, Gomez F, Zock JP. Functional and biological characteristics of asthma in cleaning workers. *Respir Med* 2013;107:673–683.
271. Arif AA, Delclos GL. Association between cleaning-related chemicals and work-related asthma and asthma symptoms among healthcare professionals. *Occup Environ Med* 2012;69:35–40.
272. Vogel CFA, Haarmann-Stemmann T. The aryl hydrocarbon receptor repressor - More than a simple feedback inhibitor of AhR signaling: Clues for its role in inflammation and cancer. *Curr Opin Toxicol* 2017;2:109–119.
273. Stockinger B, Shah K, Wincent E. AHR in the intestinal microenvironment: safeguarding barrier function. *Nat Rev Gastroenterol Hepatol* 2021;18:559–570.
274. Schiering C, Wincent E, Metidji A, Iseppon A, Li Y, Potocnik AJ, Omenetti S, Henderson CJ, Wolf CR, Nebert DW, Stockinger B. Feedback Control of AHR Signaling Regulates Intestinal Immunity. *Nature* 2017;542:242.
275. Rogers S, de Souza AR, Zago M, Iu M, Guerrina N, Gomez A, Matthews J, Baglole CJ. Aryl hydrocarbon receptor (AhR)-dependent regulation of pulmonary miRNA by chronic cigarette smoke exposure. *Sci Rep* 2017;7:40539.
276. Mohinta S, Kannan AK, Gowda K, Amin SG, Perdew GH, August A. Differential Regulation of Th17 and T Regulatory Cell Differentiation by Aryl Hydrocarbon Receptor Dependent Xenobiotic Response Element Dependent and Independent Pathways. *Toxicol Sci* 2015;145:233–243.
277. Poulain-Godefroy O, Bouté M, Carrard J, Alvarez-Simon D, Tsicopoulos A, de Nadai P. The Aryl Hydrocarbon Receptor in Asthma: Friend or Foe? *Int J Mol Sci* 2020;21:1–21.
278. Heijink IH, Brandenburg SM, Postma DS, Van Oosterhout AJM. Cigarette smoke impairs airway epithelial barrier function and cell–cell contact recovery. *Eur Respir J* 2012;39:419–428.

279. Tatsuta M, Kan-O K, Ishii Y, Yamamoto N, Ogawa T, Fukuyama S, Ogawa A, Fujita A, Nakanishi Y, Matsumoto K. Effects of cigarette smoke on barrier function and tight junction proteins in the bronchial epithelium: Protective role of cathelicidin LL-37. *Respir Res* 2019;20:1–14.
280. Sugier PE, Sarnowski C, Granell R, Laprise C, Ege MJ, Margaritte-Jeannin P, Dizier MH, Minelli C, Moffatt MF, Lathrop M, Cookson WOCM, Henderson AJ, von Mutius E, Kogevinas M, Demenais F, Bouzigon E. Genome-wide interaction study of early-life smoking exposure on time-to-asthma onset in childhood. *Clin Exp Allergy* 2019;49:1342–1351.
281. Takahashi K, Pavlidis S, Ng Kee Kwong F, Hoda U, Rossios C, Sun K, Loza M, Baribaud F, Chanez P, Fowler SJ, Horvath I, Montuschi P, Singer F, Musial J, Dahlen B, Dahlen S-E, Krug N, Sandstrom T, Shaw DE, Lutter R, Bakke P, Fleming LJ, Howarth PH, Caruso M, Sousa AR, Corfield J, Auffray C, De Meulder B, Lefaudeux D, *et al.* Sputum proteomics and airway cell transcripts of current and ex-smokers with severe asthma in U-BIOPRED: an exploratory analysis. *Eur Respir J* 2018;51:1702173.
282. Gerday S, Schleich F, Henket M, Guissard F, Paulus V, Louis R. Revisiting differences between atopic and non-atopic asthmatics: When age is shaping airway inflammatory profile. *World Allergy Organ J* 2022;15:100655.
283. Mi H, Muruganujan A, Ebert D, Huang X, Thomas PD. PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. *Nucleic Acids Res* 2019;47:D419.
284. Papi A, Brightling C, Pedersen SE, Reddel HK. Asthma. *Lancet (London, England)* 2018;391:783–800.
285. Beaven MA. Our perception of the mast cell from Paul Ehrlich to now. *Eur J Immunol* 2009;39:11–25.
286. Thapaliya M, Chompunud Na Ayudhya C, Amponnawarat A, Roy S, Ali H. Mast Cell-Specific MRGPRX2: a Key Modulator of Neuro-Immune Interaction in Allergic Diseases. *Curr Allergy Asthma Rep* 2021;21:1–19.
287. Pin I, Gibson PG, Kolendowicz R, Girgis-Gabardo A, Denburg JA, Hargreave FE, Dolovich J, Gibson PG, Kolendowicz R, Girgis-Gabardo A, Denburg JA, Hargreave FE, Dolovich J, Dolovich D. Use of induced sputum cell counts to investigate airway inflammation in asthma. *Thorax* 1992;47:25–29.
288. Wang G, Baines KJ, Fu JJ, Wood LG, Simpson JL, McDonald VM, Cowan DC, Taylor DR, Cowan JO, Gibson PG. Sputum mast cell subtypes relate to eosinophilia and corticosteroid response in asthma. *Eur Respir J* 2016;47:1123–1133.
289. Tiotiu A, Badi Y, Kermani NZ, Sanak M, Kolmert J, Wheelock CE, Hansbro PM, Dahlén SE, Sterk PJ, Djukanovic R, Guo Y, Mumby S, Adcock IM, Chung KF, Hoda U, Rossios C, Bel E, Rao N, Myles D, Compton C, Van Geest M, Howarth P, Roberts G, Lefaudeux D, De Meulder B, Bansal AT, Knowles R, Erzen D, Wagers S, *et al.* Association of Differential Mast Cell Activation with Granulocytic Inflammation in Severe Asthma. *Am J Respir Crit Care Med* 2022;205:397–411.
290. Fricker AM, Qin L, Niessen N, Katherine J, Fricker M, Qin L, Niessen N, Baines KJ, McDonald VM, Scott HA, Simpson JL, Gibson PG. Relationship of sputum mast cells with clinical and inflammatory characteristics of asthma. *Clin Exp Allergy* 2020;50:696–707.
291. Winter NA, Qin L, Gibson PG, McDonald VM, Baines KJ, Faulkner J, Evans T-J, Fricker M. Sputum mast cell/basophil gene expression relates to inflammatory and clinical features of severe asthma. *J Allergy Clin Immunol* 2021;148:428–438.
292. Murphy RC, Chow YH, Lai Y, Al-Shaikhly T, Petroni DH, Black M, Hamerman JA, Lacy-Hulbert A, Piliponsky AM, Hallstrand TS. Identification of mast cell progenitor cells in the airways of individuals with allergic asthma. *Allergy* 2023;78:547–549.
293. Hvidtfeldt M, Sverrild A, Pulga A, Frøssing L, Silberbrandt A, Hostrup M, Thomassen M, Sanden C, Clausson CM, Siddhuraj P, Bornesund D, Nieto-Fontarigo JJ, Uller L, Erjefält J, Porsbjerg C. Airway Hyperresponsiveness Reflects Corticosteroid-Sensitive Mast Cell Involvement Across Asthma Phenotypes. *J Allergy Clin Immunol* 2023;0:
294. León B, Ballesteros-Tato A. Modulating Th2 Cell Immunity for the Treatment of Asthma. *Front Immunol* 2021;12:1–14.
295. Joos RF, Germonpre PR, Kips JC, Peleman RA, Pauwels RA. Sensory neuropeptides and the human lower airways: present state and future directions. *Eur Respir J* 1994;7:1161–1171.
296. Tomaki M, Ichinose M, Miura M, Hirayama Y, Yamauchi H, Nakajima N, Shirato K. Elevated substance P content in induced sputum from patients with asthma and patients with chronic bronchitis. *Am J Respir Crit Care Med* 1995;151:613–617.
297. Xu H, Shi X, Li X, Zou J, Zhou C, Liu W, Shao H, Chen H, Shi L. Neurotransmitter and neuropeptide regulation of mast cell function: a systematic review. *J Neuroinflammation 2020 171* 2020;17:1–15.
298. Steinhoff MS, von Mentzer B, Geppetti P, Pothoulakis C, Bunnett NW. Tachykinins and their receptors: contributions to physiological control and the mechanisms of disease. *Physiol Rev* 2014;94:265–301.
299. Domingo C, Palomares O, Sandham DA, Erpenbeck VJ, Altman P. The prostaglandin D2 receptor 2 pathway in asthma: A key player in airway inflammation 11 Medical and Health Sciences 1107 Immunology 11 Medical and Health Sciences 1102 Cardiorespiratory Medicine and Haematology. *Respir Res* 2018;19:1–8.
300. Woolhouse IS, Bayley DL, Stockley RA. Effect of sputum processing with dithiothreitol on the detection of inflammatory mediators in chronic bronchitis and bronchiectasis. *Thorax* 2002;57:667–671.
301. Dahlin JS, Malinovschi A, Öhrvik H, Sandelin M, Janson C, Alving K, Hallgren J. Lin- CD34hi CD117int/hi FcεRI+ cells in human blood constitute a rare population of mast cell progenitors. *Blood* 2016;127:383–91.
302. Galli SJ, Tsai M. IgE and mast cells in allergic disease. *Nat Med* 2012;18:693.
303. Min H, Montecino-Rodriguez E, Dorshkind K. Effects of aging on early B- and T-cell development. *Immunol Rev* 2005;205:7–17.
304. Möllerherm H, Meier K, Schmies K, Fuhrmann H, Naim HY, von Köckritz-Blickwede M, Branitzki-Heinemann K.

Differentiation and Functionality of Bone Marrow-Derived Mast Cells Depend on Varying Physiologic Oxygen Conditions. *Front Immunol* 2017;8:113–122.
305. Saito H. Culture of human mast cells from hemopoietic progenitors. *Methods Mol Biol* 2006;315:113–22.
306. Cop N, Ebo DG, Bridts CH, Elst J, Hagendorens MM, Mertens C, Faber MA, Clerck LS De, Sabato V, De Clerck LS, Sabato V. Influence of IL-6 , IL-33 , and TNF- a on Human Mast Cell Activation : Lessons from Single Cell Analysis by Flow Cytometry. *Cytom Part B Clin Cytom* 2018;94:405–411.
307. Houdt VM, Gasse V AL, Tendeloo V V, J-p T. MRGPRX2 and Immediate Drug Hypersensitivity: Insights From Cultured Human Mast Cells. *J Investig Allergol Clin Immunol* 2021;31:489–499.
308. O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP, Shanahan F. The role of substance P in inflammatory disease. *J Cell Physiol* 2004;201:167–180.
309. Porebski G, Kwiecien K, Pawica M, Kwitniewski M. Mas-Related G Protein-Coupled Receptor-X2 (MRGPRX2) in Drug Hypersensitivity Reactions. *Front Immunol* 2018;9:3027.
310. Holgate ST, Finnerty JP. Antihistamines in asthma. *J Allergy Clin Immunol* 1989;83:537–547.
311. Bernstein IL. Cromolyn sodium in the treatment of asthma: coming of age in the United States. *J Allergy Clin Immunol* 1985;76:381–8.
312. Couto M, Moreira A, Delgado L. Diagnosis and treatment of asthma in athletes. *Breathe* 2012;8:286–296.
313. Becker JM, Rogers J, Rossini G, Mirchandani H, D'Alonzo GE. Asthma deaths during sports: Report of a 7-year experience. *J Allergy Clin Immunol* 2004;113:264–267.
314. Dweik RA, Boggs PB, Erzurum SC, Irvin CG, Leigh MW, Lundberg JO, Olin A-C, Plummer AL, Taylor DR, American Thoracic Society Committee on Interpretation of Exhaled Nitric Oxide Levels (FENO) for Clinical Applications. An official ATS clinical practice guideline: interpretation of exhaled nitric oxide levels (FENO) for clinical applications. *Am J Respir Crit Care Med* 2011;184:602–15.
315. Motomura C, Matsuzaki H, Odajima H, Oki T, Yasunari Y, Kawano T, Iwata M, Okabe K, Wakatsuki M, Murakami Y, Taba N, Honjo S, Ohga S. Effect of age on exercise-induced bronchoconstriction in children and adolescents with asthma. *J Asthma* 2022;59:297–305.
316. Kim K, Cho HJ, Yoon JW, Choi SH, Sheen YH, Han MY, Baek H. Exhaled nitric oxide and mannitol test to predict exercise-induced bronchoconstriction. *Pediatr Int* 2018;60:691–696.
317. Chinellato I, Piazza M, Peroni D, Sandri M, Chiorazzo F, Boner AL, Piacentini G. Bronchial and alveolar nitric oxide in exercise-induced bronchoconstriction in asthmatic children. *Clin Exp Allergy* 2012;42:1190–1196.
318. Dumas O, Bédard A, Marbac M, Sedki M, Temam S, Chanoine S, Severi G, Boutron-Ruault MC, Garcia-Aymerich J, Siroux V, Varraso R, Le Moual N. Household Cleaning and Poor Asthma Control Among Elderly Women. *J allergy Clin Immunol Pract* 2021;9:2358-2365.e4.
319. Riaz F, Pan F, Wei P. Aryl hydrocarbon receptor: The master regulator of immune responses in allergic diseases. *Front Immunol* 2022;13:7672.
320. Schmidt J V, Bradfield CA. AH RECEPTOR SIGNALING PATHWAYS. *Annu Rev Cell Dev Biol* 1996;12:55–89.
321. Veldhoen M, Hirota K, Westendorf AM, Buer J, Dumoutier L, Renauld JC, Stockinger B. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* 2008;453:106–109.
322. Stämpfli MR, Anderson GP. How cigarette smoke skews immune responses to promote infection, lung disease and cancer. *Nat Rev Immunol 2009 95* 2009;9:377–384.
323. Sopori M. Effects of cigarette smoke on the immune system. *Nat Rev Immunol 2002 25* 2002;2:372–377.
324. Heijink IH, Brandenburg SM, Postma DS, Van Oosterhout AJM. Cigarette smoke impairs airway epithelial barrier function and cell-cell contact recovery. *Eur Respir J* 2012;39:419–428.
325. Sohail MU, Al-Mansoori L, Al-Jaber H, Georgakopoulos C, Donati F, Botrè F, Sellami M, Elrayess MA. Assessment of Serum Cytokines and Oxidative Stress Markers in Elite Athletes Reveals Unique Profiles Associated With Different Sport Disciplines. *Front Physiol* 2020;11:1317.
326. Bougault V, Adami PE, Sewry N, Fitch K, Carlsten C, Villiger B, Schwellnus M, Schobersberger W. Environmental factors associated with non-infective acute respiratory illness in athletes: A systematic review by a subgroup of the IOC consensus group on "acute respiratory illness in the athlete." *J Sci Med Sport* 2022;25:466–473.
327. Ventura LMB, Ramos MB, Gioda A, França BB, de Oliveira Godoy JM. Air quality monitoring assessment during the 2016 Olympic Games in Rio de Janeiro, Brazil. *Environ Monit Assess* 2019;191:1–14.
328. For a healthy Belgium. Air quality - For a Healthy Belgium. 2022;at <https://www.healthybelgium.be/en/health-status/determinants-of-health/air-quality>.
329. Traboulsi H, de Souza AR, Allard B, Haidar Z, Sorin M, Moarbes V, Fixman ED, Martin JG, Eidelman DH, Baglole CJ. Differential Regulation of the Asthmatic Phenotype by the Aryl Hydrocarbon Receptor. *Front Physiol* 2021;12:720196.
330. Forsythe P. Mast Cells in Neuroimmune Interactions. *Trends Neurosci* 2019;42:43–55.
331. Jensen BM, Frandsen PM, Raaby EM, Schiøtz PO, Skov PS, Poulsen LK. Molecular and stimulus-response profiles illustrate heterogeneity between peripheral and cord blood-derived human mast cells. 2012;doi:10.1189/jlb.0712354.
332. Dahl C, Hoffmann HJ, Saito H, Schiøtz PO. Human mast cells express receptors for IL-3, IL-5 and GM-CSF; a partial map of receptors on human mast cells cultured in vitro. *Allergy* 2004;59:1087–1096.
333. L'homme L, Dombrowicz D. In vitro models of human mast cells: How to get more and better with induced pluripotent stem cells? *J Allergy Clin Immunol* 2022;149:904–906.

334. Martens K, Hellings PW, Steelant B. Calu-3 epithelial cells exhibit different immune and epithelial barrier responses from freshly isolated primary nasal epithelial cells in vitro. *Clin Transl Allergy* 2018;8:40.
335. Falquet M, Ercolano G, Jandus P, Jandus C, Trabanelli S. Healthy and Patient Type 2 Innate Lymphoid Cells are Differently Affected by in vitro Culture Conditions. *J Asthma Allergy* 2021;14:773–783.
336. de Lucía Finkel P, Sherwood C, Saranchova I, Xia W, Munro L, Pfeifer CG, Piret JM, Jefferies WA. Serum free culture for the expansion and study of type 2 innate lymphoid cells. *Sci Reports 2021 111* 2021;11:1–10.

Summary

Summary

Human airways are continuously exposed to external triggers trough breathing, which can initiate epithelial damage. This may induce an inflammatory response, resulting in bronchoconstriction. It is known that in asthma, which is an heterogenous disease characterized by reversible airway obstruction, there is a complex interaction between airway epithelium and immune cells in the initiation and continuation of airway inflammation. As mast cells are located close the airway epithelium, we hypothesize they are critical in mediating this response. Released mast cell mediators via both IgE- and IgE-independent mast cell activation are able to induce bronchoconstriction. The role of the newly described MRGPRX2 receptor in this cascade is not fully understood. Besides, bronchoconstriction can also occur in otherwise healthy subjects. This phenomenon is called exercise-induced bronchoconstriction (EIB). Athletes have an increased risk to develop EIB, which is described to be associated with sport intensity and additional external triggers such as cold air in cross country skiing or chlorine by-products in swimmers. Even adolescent athletes, at the start of their professional career, have increased risk to develop EIB, highlighting the need to better identify these athletes at risk for EIB.

The main objective of this thesis was to study airway inflammation as a result of external triggers inducing epithelial damage. We hypothesize that adolescent athletes are more vulnerable to external triggers due to increased ventilation rate, which might act as stressor to the airway barrier. Therefore, the first aim was to study atopy and EIB in intense adolescent athletes. Secondly, we analysed more in depth the effect of external stimuli on the airways of elite adolescent athletes and asthmatics. Finally, we studied the central role of mast cells in airway inflammation.

Atopy has been significantly associated with bronchial hyperreactivity and EIB in adult elite athletes. Therefore, a screening tool may help with the early identification of atopy and allergy symptom development, which may impact physical performances in adolescent athletes. In chapter 4, AQUA© score of ≥ 6 and FeNO levels of ≥ 15 ppb were identified as prediction tool for EIB in adolescent elite athletes. These results were confirmed in recreational athletes performing at least 12 hours of sport a week (chapter 5). Our study showed the presence of atopy in approximately 40% of adolescent athletes in both cohorts, which is higher than in the general population. Furthermore, 14% of recreational athletes reported previous asthma

diagnosis and 22% of recreational tested positive for EIB. Of these EIB^+ athletes, 76% of athletes did not receive a prior asthma diagnosis, which is often used interchangeably in real life practise. These results indicate the need to better identify EIB in adolescent athletes. Investigating different factors linked to EIB, the highest sensitivity was found for AQUA© ≥ 6 and highest specificity was found for reporting wheeze during exercise. Furthermore, previous asthma diagnosis was associated with outdoor athletes, highlighting the impact of the environment during intense exercise. Serum levels of epithelial damage were not able to differentiate EIB^+ and EIB^- athletes, but were associated to training type, training intensity and EIB severity.

In chapter 6, we investigated the effect of intense exercise and environmental exposure to air pollution on the airways of adolescent elite athletes. Indeed, RNA-Seq analysis of sputum transcriptome showed significantly differentially expressed genes in athletes compared with controls, which were related to inflammation and epithelial cell damage. In addition, sputum samples of athletes contained significantly more carbon loaded airway macrophages compared with controls, likely the result of their high ventilatory demands during exercise. In addition, significantly lower mRNA levels of OCLN and ZO-1 in athletes exposed to higher PM_{10} levels compared with athletes exposed to lower levels were observed. Remarkably, the airway response to EVH testing in athletes was associated to prior PM exposure, indicating that exposure to increased air pollution may induce short term increased airway hyperreactivity. Our preliminary RNA-Seq analysis between EIB^+ and EIB^- athletes suggested a role of epithelial damage, oxidative stress and (neuro)inflammation in EIB.

In chapter 7, a retrospective analysis was performed of environmental exposures of patients with asthma, including smoking and work-related exposures. We demonstrated increased epithelial damage in asthmatic patients compared with healthy controls, suggesting that they might be more vulnerable for external triggers. We indeed found significant differences amongst sputum transcriptome of asthmatics exposed to cigarette smoke or work-related exposure to cleaning products compared with asthmatic patients without exposure. A role for the aryl hydrocarbon pathway (AhR) for airway inflammation in asthmatic patients exposed to irritants was suggested.

Lastly, the involvement of mast cells in non-IgE mediated airway inflammation was investigated. In chapter 8, a pilot study was performed in asthmatic patients compared with healthy controls to characterize MRGPRX2 expressing mast cells in sputum samples. Sputum

mast cells were increased in allergic asthmatic patients compared with controls. However, also increased mast cell activation was observed in non-allergic asthma. MRGPRX2 expression was not associated with allergic or non-allergic asthma phenotype. Furthermore, neuromediator NKA correlated positively with the percentage of mast cells and negatively with tiffeneau index. These results suggested a role for mast cell in neuro-immune reaction for both allergic and non-allergic asthma patients. To better investigate this role of mast cell, a human mast cell differentiation protocol was optimized to obtain functional MRGPRX2 expressing mast cells in chapter 9. Stimulation of mast cells with substance P resulted in increased CD63 expression and the classical inhibitor ketotifen was able to inhibit this activation. The optimized *in vitro* model can be used to explore the role of mast cell in especially MRGPRX2 mediated activation and as screening tool for potential therapeutics.

To conclude, we showed that intense adolescent athletes had increased markers of environmental exposure (such as air pollution) and epithelial damage. This confirms the need to proper follow-up these athletes, which can be provided by proposed screening tools. Furthermore, also asthmatic subjects showed impact of external triggers on their sputum transcriptome. Regarding the underlying response to external triggers inducing epithelial damage, a role for non-IgE mediated mast cell activation is proposed. Within this thesis, research tools investigating MRGPRX2 mediated activation were developed.

Samenvatting

De menselijke luchtwegen worden door ademhaling voortdurend blootgesteld aan externe triggers die epitheelschade kunnen veroorzaken. Dit kan leiden tot verstoring van weefselhomeostase en een ontstekingsreactie veroorzaken, met bronchoconstrictie als gevolg. Astma is een heterogene ziekte gekenmerkt door omkeerbare luchtwegobstructie. Het is bekend dat bij de initiatie en voortzetting van deze luchtwegontsteking er een complexe interactie bestaat tussen luchtwegepitheel en immuuncellen. Aangezien mestcellen zich dicht bij het luchtwegepitheel bevinden, veronderstellen wij dat zij van cruciaal belang zijn voor het mediëren van deze respons. Vrijgekomen mestcelmediatoren via zowel IgE-afhankelijke als IgE-onafhankelijke mestcelactivatie kunnen bronchoconstrictie induceren. De rol van de onlangs beschreven MRGPRX2-receptor in deze cascade is niet volledig begrepen. Bovendien kan bronchoconstrictie ook optreden bij overigens gezonde personen. Dit verschijnsel wordt inspanningsgeïnduceerde bronchoconstrictie (EIB) genoemd. Atleten hebben een verhoogd risico op EIB, dat in verband wordt gebracht met de intensiteit van de sport en bijkomende externe triggers zoals koude lucht bij langlaufen of chloor-bijproducten bij zwemmers. Zelfs adolescenten aan het begin van hun professionele carrière hebben een verhoogd risico om EIB te ontwikkelen, wat de noodzaak benadrukt om deze atleten met een risico op EIB beter te identificeren.

Het hoofddoel van dit proefschrift was het bestuderen van luchtwegontsteking als gevolg van externe triggers die epitheliale schade veroorzaken. Onze hypothese is dat adolescente sporters kwetsbaarder zijn voor externe triggers vanwege de verhoogde ventilatie die als stressor voor de luchtwegbarrière zou kunnen werken. Daarom was het eerste doel atopie en EIB te bestuderen bij intensieve adolescente sporters. Ten tweede analyseerden we grondiger het effect van externe prikkels op de luchtwegen van adolescente topsporters en astmapatiënten. Ten slotte bestudeerden we de centrale rol van mestcellen in luchtwegontsteking.

Atopie is significant geassocieerd met bronchiale hyperreactiviteit en EIB bij volwassen topsporters. Daarom kan een screeningsinstrument helpen bij de vroege identificatie van atopie en de ontwikkeling van allergiesymptomen die de fysieke prestaties van adolescente atleten reeds kunnen beïnvloeden. In hoofdstuk 4 werden AQUA© score van ≥ 6 en FeNO waarde van ≥ 15 ppb geïdentificeerd als screening instrument voor EIB bij adolescente

topsporters. Deze resultaten werden bevestigd bij recreatieve sporters die minstens 12 uur per week sporten (hoofdstuk 5). Onze studie toonde de aanwezigheid van atopie aan bij ongeveer 40% van de adolescente sporters in beide cohorten, wat hoger is dan in de algemene bevolking. Verder meldde 14% van de recreationele sporters een eerdere diagnose van astma en 22% van de recreationele sporters testte positief op EIB. Van deze EIB^+ atleten werd 80% niet behandeld met een inhalatietherapie. Deze resultaten wijzen op de noodzaak van een betere identificatie van EIB bij adolescente sporters. Bij onderzoek van verschillende factoren die verband houden met EIB werd de hoogste sensitiviteit gevonden voor AQUA© ≥ 6 en de hoogste specificiteit werd gedetecteerd voor het melden van piepen tijdens inspanning. Bovendien werd een eerdere diagnose van astma geassocieerd met buitensporters, wat de invloed van de omgeving tijdens intensieve inspanning benadrukt. Serum waardes van epitheelschade konden geen onderscheid maken tussen EIB^+ en EIB^- atleten, maar waren wel geassocieerd met het type training, de trainingsintensiteit en de ernst van EIB.

In hoofdstuk 6 onderzochten we het effect van intensieve inspanning en blootstelling aan luchtvervuiling op de luchtwegen van adolescente topsporters. RNA-Seq analyse van het sputum transcriptoom toonde inderdaad significant differentieel geëxpresseerde genen bij atleten in vergelijking met controles die gerelateerd waren aan ontsteking en epitheliale cel schade. Bovendien bevatten sputum stalen van atleten significant meer koolstof beladen luchtwegmacrofagen dan controles. Dit is waarschijnlijk het gevolg van hun hoge ventilatie tijdens het sporten. Bovendien werden significant lagere mRNA-niveaus van OCLN en ZO-1 waargenomen bij atleten blootgesteld aan hogere PM_{10}-waardes in vergelijking met atleten blootgesteld aan lagere niveaus. Opmerkelijk is dat de luchtwegrespons op EVH-tests bij atleten samenhing met eerdere blootstelling aan PM_{10}, wat erop wijst dat blootstelling aan verhoogde luchtvervuiling op korte termijn een verhoogde hyperreactiviteit van de luchtwegen kan veroorzaken. Onze preliminaiere RNA-Seq analyse tussen EIB^+ en EIB^- atleten suggereerde een rol van epitheliale schade, oxidatieve stress en (neuro)inflammatie bij EIB.

In hoofdstuk 7 werd een retrospectieve analyse uitgevoerd van milieublootstellingen van patiënten met astma, waaronder roken en werk-gerelateerde blootstellingen. Wij toonden verhoogde epitheelschade aan bij astmapatiënten in vergelijking met gezonde controles, wat suggereert dat zij kwetsbaarder zouden kunnen zijn voor externe triggers. Wij vonden inderdaad significante verschillen in het transcriptoom van sputumstalen van astmapatiënten blootgesteld aan sigarettenrook of werkgerelateerde blootstelling aan schoonmaakmiddelen

in vergelijking met astmapatiënten zonder blootstelling. Een rol voor de aryl koolwaterstofroute (AhR) bij luchtwegontsteking bij astmapatiënten die zijn blootgesteld aan irriterende stoffen werd gesuggereerd.

Tenslotte werd de betrokkenheid van mestcellen bij niet-IgE gemedieerde luchtwegontsteking onderzocht. In hoofdstuk 8 werd een proefonderzoek uitgevoerd bij astmapatiënten in vergelijking met gezonde controles om mestcellen met MRGPRX2 in sputumstalen te karakteriseren. Sputum mestcellen waren verhoogd bij allergische astmapatiënten in vergelijking met gezonde controles. Maar ook bij niet-allergische astmapatiënten werd een verhoogde mestcelactivatie waargenomen. MRGPRX2 expressie was niet geassocieerd met allergisch of niet-allergisch astma fenotype. Bovendien correleerde neuromediator NKA positief met het percentage mestcellen en negatief met de tiffeneau-index. Deze resultaten suggereerden een rol voor mestcellen in de neuro-immuunreactie bij zowel allergische als niet-allergische astmapatiënten. Om deze rol van mestcellen beter te onderzoeken, werd in hoofdstuk 9 een menselijk mestceldifferentiatieprotocol geoptimaliseerd om functionele MRGPRX2 expresserende cellen te verkrijgen. Stimulatie van mestcellen met substance P resulteerde in verhoogde CD63 expressie en de klassieke inhibitor ketotifen kon deze activatie remmen. Het geoptimaliseerde *in vitro* model kan worden gebruikt om de rol van mestcellen, met name MRGPRX2 gemedieerde activering te onderzoeken en als screeningsinstrument voor potentiële therapeutica.

In conclusie toonden wij aan dat intensieve adolescente sporters verhoogde markers van externe triggers (zoals luchtvervuiling) en epitheelschade hadden. Dit bevestigt de noodzaak van een goede follow-up van deze atleten, in welke de opgestelde screeningsmogelijkheden toegepast kunnen worden. Bovendien was ook in astmapatiënten de impact van externe triggers op hun sputum transcriptoom waarneembaar. Met betrekking tot de onderliggende reactie op externe triggers die epitheliale schade veroorzaken, wordt ook een rol voor niet-IgE-gemedieerde mestcelactivatie voorgesteld. Binnen dit proefschrift werden onderzoeks-tools ontwikkeld die MRGPRX2 gemedieerde activatie onderzoeken.

Scientific acknowledgments, personal contribution and conflict of interest

Scientific acknowledgements

We want to thank all patients, athletes and healthy controls who have participated in our studies. Furthermore, we thank the 'Elite sports' schools' of Antwerp, Leuven and Vilvoorde for their cooperation. In addition, we thank our colleagues from the Allergy and Clinical Immunology Research Group, Ahmad Kasran, Backaert Wout, Staels Frederik and Ieven Toon to supervise as doctor the visits at the 'Elite sports' schools'. Moreover, we highly appreciate the help of the Clinical Department of Pneumology, for the ability to use their equipment for lung function measurements and for training us to use these systems. W. Bossuyt, V. Brys, A. Cortés Calabuig and the UZ Leuven Genomics Core are acknowledged for RNA-Seq analysis and bioinformatics expertise. We are very grateful to C. Wang of the Centre for Environmental Sciences, Hasselt University, for sharing expertise and critical insights. Also thanks to Ellen Dilissen and Jonathan Cremer for their technical help with the flow cytometric experiments, RT-qPCR and protein analyses. We would also like to mention Liesbeth Van Noten and Imke Wens for their contribution during their internship to the *in vitro* part of this thesis.

Personal contributions

Chapters 1, 3 and 10 were written by Goossens J and revised by Dupont L and Bullens D.

Chapter 2 was written by Goossens J and critically revised by all authors.

Chapter 4 was written by Goossens J and critically revised by all authors. The study was conceptualized and designed by Goossens J, Raes M, Verelst S, Leus J, Dupont L and Bullens D. Goossens J coordinated the study visits at 'Elite Sport Schools' in Flanders. Goossens J, Jonckheere AC, Dilissen E, Vanbelle V, Aergeerts S, Stappers J, Peers K contributed to the recruitment of athletes and/or performing of clinical assessments. Goossens J analyzed and interpreted the results. Vandekerckhove J, Jonckheere AC, Seys S, Dupont L and Bullens D also contributed to interpretation of results.

Chapter 5 was written by Goossens J and revised by Vandekerckhove J, Jonckheere AC, Bullens D and Dupont L. The study was conceptualized and designed by De Wilde B, Leus J, Verelst S, Raes M, Dupont L and Bullens D. Goossens J performed the recruitment of athletes and performed the clinical assessments in UZ Leuven with help of Vandekerckhove J and Dilissen E. De Wilde B and Leus J organized the clinical assessments of athletes in AZ Maria Middelares Ghent. Verelst S and Raes M organized the clinical assessments in Jessa Hospital Hasselt. Processing and analysis of samples was performed by Goossens J and Dilissen E.

Goossens J performed the analysis and interpretation of the results. Vandekerckhove J, Jonckheere AC, Dupont L and Bullens D contributed to the interpretation of the results.

Chapter 6 was written by Goossens J and critically revised by all authors. The study was conceptualized and designed by Raes M, Verelst S, Leus J, Dupont L and Bullens D. Goossens J coordinated the study visits at 'Elite Sport Schools' in Flanders. Goossens J, Jonckheere AC, Dilissen E, Goossens C, Vanbelle V, Aergeerts S, Stappers J, Peers K contributed to the recruitment of athletes and/or performing of clinical assessments. Goossens J performed the analysis and interpretation of results (including RNA-Seq interpretation). Jonckheere AC, Seys S, Decaesteker T, Dupont L and Bullens D contributed to the interpretation of the results.

Chapter 7 was written by Goossens J and Jonckheere AC and critically revised by all authors. De Boodt S collected retrospectively clinical characteristics of included patients. qPCR was performed by Dilissen E. Protein assays were performed by Goossens J, Jonckheere AC and Dilissen E. RNA-Seq analysis interpretation was performed by Goossens J and Jonckheere AC. Goossens J and Jonckheere AC performed the analysis and interpretation of results. Seys S, Marain F, Dupont L and Bullens D contributed to the interpretation of the results.

Chapter 8 was written by Goossens J and revised by Jonckheere AC, Dupont L and Bullens D. The study was conceptualized by Bullens D. Goossens J performed the recruitment of participants, clinical assessments and processing of the samples. Goossens J, Jonckheere AC and Cremer J contributed to the development of flow cytometric panel. Cremer J ran the provided samples on the flow cytometer and data were analysed by Goossens J. Goossens J performed the analysis and interpretation of results.

Chapter 9 was written by Goossens J and revised by Ieven T, Jonckheere AC, Dupont L and Bullens D. Goossens J, Ieven T and Bullens D conceptualized the study. Donor samples were collected by Goossens J via Red Cross and by Goossens J and Ieven T via recruitment of donors. Blood sampling was performed by Goossens J and Ieven T. *In vitro* experiments were conducted by Goossens J and Ieven T with help of Dilissen E, Jonckheere AC and Roossens W. Cremer J ran the provided samples on flow cytometer and data were analysed by Ieven T. Goossens J and Ieven T performed analysis and interpretation of results.

Conflicts of interest

There are no conflicts of interest.

Funding

Bullens Dominique and Dupont Lieven have a FWO senior fellowship. The work of chapter 2, 4, 5 and 6 was supported by an applied Biomedical Research with a Primary Social finality project (TBM) grant of the Flemish government, Belgium (T001417N).

Publications

Publications in peer-reviewed journals

First author

1. **Janne Goossens**, Anne-Charlotte Jonckheere, Sven F Seys, Ellen Dilissen, Tatjana Decaesteker, Camille Goossens, Koen Peers, Vincent Vanbelle, Jeroen Stappers, Sven Aertgeerts, Barbara De Wilde, Jasmine Leus, Sophie Verelst, Marc Raes, Lieven Dupont, Dominique MA Bullens. Activation of epithelial and inflammatory pathways in adolescent elite athletes exposed to intense exercise and air pollution. Thorax. March 2023. doi: 10.1136/thorax-2022-219651.

2. **Janne Goossens**, Josefien Vandekerckhove, Anne-Charlotte Jonckheere, Ellen Dilissen, Sven F Seys, Vincent Vanbelle, Sven Aertgeerts, Jeroen Stappers, Koen Peers, Marc Raes, Sophie Verelst, Jasmine Leus, Lieven J. Dupont, Dominique MA Bullens. Can AQUA© questionnaire and FeNO predict atopy in early-career athletes? Pediatric Allergy and Immunology. March 2023. doi: 10.1111/pai.13936.

3. **Janne Goossens,** Dominique MA Bullens, Lieven J Dupont, Sven F Seys. Exposome mapping in chronic respiratory diseases: the added value of digital technology. Curr Opin Allergy Clin Immunol. Feb 2022. doi: 10.1097/ACI.0000000000000801.

4. **Janne Goossens,** Tatjana Decaesteker, Anne-charlotte Jonckheere, Sven Seys, Sophie Verelst, Lieven Dupont, Dominique MA Bullens. How to detect young athletes at risk of exercise-induced bronchoconstriction? Paediatr Respir Rev. Dec 2021. doi: 10.1016/j.prrv.2021.09.007.

5. **Janne Goossens*,** Anne-Charlotte Jonckheere*, Lieven J. Dupont, Dominique M. A. Bullens. Air Pollution and the Airways: Lessons from a Century of Human Urbanization. Atmosphere. July 2021. doi: 10.3390/atmos12070898. *Contributed equally

Co-author

1. Ganseman E, **Goossens J**, Blanter M, Jonckheere AC, Bergmans N, Vanbrabant L, Gouwy M, Ronsmans S, Vandenbrouck S, Dupont L, Vanoirbeek J, Bullens D, Breynaert C, Proost P, Schrijvers R. Frequent allergic sensitization to farmed edible insects in exposed employees. The Journal of Allergy and Clinical Immunology: In Practice. *Under review.*

2. Jonckheere AC, Seys S, Dilissen E, Schelpe AS, Van der Eycken S, Corthout S, Verhalle T, **Goossens J**, Vanbelle V, Aertgeerts S, Troosters T, Peers K, Dupont L, Bullens D. Early-onset airway damage in early-career elite athletes: A risk factor for exercise-induced bronchoconstriction. J Allergy Clin Immunol. 2019.

3. De Schaepdryver M, **Goossens J**, De Meyer S, Jeromin A, Masrori P, Brix B, Claeys KG, Schaeverbeke J, Adamczuk K, Vandenberghe R, Van Damme P, Poesen K. Serum neurofilament heavy chains as early marker of motor neuron degeneration. Ann Clin Transl Neurol. 2019.

4. Gille B, De Schaepdryver M, Dedeene L, **Goossens J**, Claeys KG, Van Den Bosch L, Tournoy J, Van Damme P, Poesen K. Inflammatory markers in cerebrospinal fluid: independent prognostic biomarkers in amyotrophic lateral sclerosis? J Neurol Neurosurg Psychiatry. 2019.

5. De Schaepdryver M, **Goossens J**, Jeromin A, Brix B, Van Damme P, Poesen K. Analytical performance of a CE-marked immunoassay to quantify phosphorylated neurofilament heavy chains. Clinical Chemistry and Laboratory Medicine. 2019.

6. Gille B, De Schaepdryver M, **Goossens J**, Dedeene L, De Vocht J, Oldoni E, Goris A, Van Den Bosch L, Depreitere B, Claeys KG, Tournoy J, Van Damme P, Poesen K. Serum neurofilament light chain levels as a marker of upper motor neuron degeneration in patients with Amyotrophic Lateral Sclerosis. Neuropathology and Applied Neurobiology. 2018.

In preparation

1. **Goossens J,** Vandekerckhove J, Jonckheere AC, De Wilde B, Leus J, Verelst S, Raes M, Dupont L, Bullens D. Exercise-induced bronchoconstriction in recreational adolescent athletes.

2. **Goossens J***, Jonckheere AC*, De Boodt S, Dilissen E, Marain F, Dupont L, Seys S, Bullens D. Validation of cluster analysis of sputum cytokine profiles in asthmatic patients reveals underlying molecular pathways. *Contributed equally

3. **Goossens J*,** Ieven T*, Dilissen E, Cremer J, Jonckheere AC, Roosens W, Lieven D, Bullens D. Optimization of human mast cell differentiation to study MRGPRX2-induced activation in vitro. *Contributed equally

National and international presentations

1. European Academy of Allergy and Clinical Immunology (EAACI) congress 2023, Hamburg. **Goossens J**, Jonckheere AC, Dilissen E, Seys S, Peers K, Vanbelle V, Aertgeerts S, Leus J, Verelst S, Raes M, Dupont L, Bullens D. Does air pollution affect the airways of early-career elite athletes? Oral presentation.

2. GSK Pulmonology awards - Belgian Respiratory Society (BeRS) 2023, Brussels. **Goossens J**, Vandekerckhove J, Jonckheere AC, Dilissen E, Marain N, Ieven T, De Wilde B, Leus J, Verelst S, Raes M, Dupont L, Bullens D. Exercise-induced bronchoconstriction in recreational adolescent athletes: potential screening strategies. Oral presentation.

3. Childhood Immunology Symposium 2023, Leuven. **Goossens J.** External triggers inducing epithelial cell damage in exercise-induced bronchoconstriction. Oral presentation.

4. Belgian Association of Pediatrics (BVK – SBP) congress 2023, Brussels.
Goossens J, Dilissen E, Jonckheere AC, Seys S, Bogaerts S, Stappers J, Vanbelle V, Aertgeerts S, Troosters T, Moustie S, Leus J, Raes M, Peers K, Dupont L, Bullens D. Does air pollution affect the airways of early-career elite athletes? Oral presentation.

5. Belgian Pneumology days - Belgian Respiratory Society (BeRS) 2022, Brussels.
   Goossens J, Dilissen E, Jonckheere AC, Seys S, Bogaerts S, Stappers J, Vanbelle V, Aertgeerts S, Troosters T, Moustie S, Leus J, Raes M, Peers K, Dupont L, Bullens D. Can FeNO and AQUA© questionnaire predict atopy in early career athletes? Oral presentation.

6. Lung Science Conference – European Respiratory Society (ERS) 2022, Estoril.
   Goossens J, Ieven T, Dilissen E, Jonckheere AC, Cremer J, Dupont L, Bullens B. Human mast cell differentiation optimization to study MRGPRX2 induced activation in vitro. Poster presentation.

7. European Academy of Allergy and Clinical Immunology (EAACI) congress 2022, Prague.
   Goossens J, Jonckheere AC, Dilissen E, Goossens C, Seys S, Peers K, Vanbelle V, Aertgeerts S, Leus J, Verelst S, Raes M, Dupont L, Bullens D. Sputum analysis of early-career elite athletes by RNA-Seq reveals underlying mechanisms of exercise-induced bronchoconstriction (EIB) pointing to epithelial damage, immune cell trafficking and airway type 17 and type 1 inflammation. Poster presentation.

8. Belgian Pneumology days - Belgian Respiratory Society (BeRS) 2021, Antwerp.
   Goossens J, Jonckheere AC, Dilissen E, Goossens C, Seys S, Peers K, Vanbelle V, Aertgeerts S, Leus J, Verelst S, Raes M, Dupont L, Bullens D. Increased airway macophage carbon load and hyperreactivity is associated to air pollution exposure in early-career elite athletes. Oral presentation.

9. GSK Pulmonology awards - Belgian Respiratory Society (BeRS) 2021, Louvain-La-Neuve.
   Goossens J, Jonckheere AC, Dilissen E, Goossens C, Seys SF, Bogaerts S, Stappers J, Vanbelle V, Aertgeerts S, Troosters T, Peers K, Leus J, Raes M, Dupont L, Bullens D. Exercise-induced bronchoconstriction in early-career athletes: how does exposure to environmental triggers affect the airways? Oral presentation.

10. European Academy of Allergy and Clinical Immunology (EAACI) congress EAACI 2020, Londen (hybrid).
    Goossens J, Goossens C, Dilissen E, Jonckheere AC, Seys S, Bogaerts S, Stappers J, Vanbelle V, Aertgeerts S, Troosters T, Moustie S, Leus J, Raes M, Peers K, Dupont L, Bullens D. FeNO and AQUA questionnaire as prediction tool for atopy in early career elite athletes. Poster presentation.

11. Symposium Recent Advances in rehabilitation of cardiorespiratory and internal diseases – Flemish Interuniversity Research for Rehabilitation in Internal diseases (FIRRI) 2020, Hasselt.
    Goossens J, Goossens C, Dilissen E, Jonckheere AC, Seys S, Bogaerts S, Stappers J, Vanbelle V, Aertgeerts S, Troosters T, Peers K, Leus J, Raes M, Dupont L, Bullens D. Intense exercise in early career elite athletes: focus on the airways. Poster prensentation.

12. European Academy of Allergy and Clinical Immunology (EAACI) congress 2019, Lisbon.
    Goossens J, Jonckheere AC, Seys S, Dilissen E, Goossens C, Schelpe A, Van Der Eycken S, Corthout S, Verhalle T, Troosters T, Peers K, Dupont L, Bullens D. Atopic status and exercise-induced bronchoconstriction in high-school elite athletes. Poster presentation.

13. Belgian Society of Allergy and Clinical Immunology (BelSACI) annual meeting 2018, Leuven. Jonckheere AC, **Goossens J***, Seys S, Dilissen E, Schelpe AS, Van der Eycken S, Verhalle T, Corthout S, Goossens C, Vanbelle V, Aertgeerts S, Troosters T, Peers K, Dupont L, Bullens D. Increased sputum uric acid levels and serum CC16 levels reveal early epithelial damage in high-school young elite athletes. Oral presentation. *Presenting author

Grants

1. GSK Clinical Science Award 2023 2nd price for the abstract 'Exercise-induced bronchoconstriction in recreational adolescent athletes: potential screening strategies' presented at the GSK Pulmonology awards 2023.

2. EAACI travel grant for the abstract 'Does air pollution affect the airways of early-career elite athletes?' presented at EAACI congress 2023.

3. FWO travel grant for the abstract 'Sputum analysis of early-career elite athletes by RNA-Seq reveals underlying mechanisms of exercise-induced bronchoconstriction (EIB) pointing to epithelial damage, immune cell trafficking and airway type 17 and type 1 inflammation.' presented at EAACI congress 2022.

4. Lung Pediatry Award 2021 for the presentation 'Increased airway macophage carbon load and hyperreactivity is associated to air pollution exposure in early-career elite athletes.' presented at Belgian Pneumology days 2021.

5. Scientific Award for best oral poster presentation for the abstract 'Intense exercise in early career elite athletes: focus on the airways' presented at the Symposium of Recent Advances in rehabilitation of cardiorespiratory and internal diseases 2020.

6. Scientific Award for best oral poster presentation for the abstract '' presented at Belgian Society for Allergy ad Clinical Immunology (BelSACI). 2018

Short curriculum vitae

Personal information

Janne Goossens
Work email: janne.goossens@kuleuven.be
Private email: janne.goossens@telenet.be
Nationality: Belgian
Date of birth: 23/03/1995

Education

- **Doctoral training in Biomedical Sciences**, 2018 – present
  KU Leuven, Department of Microbiology, Immunology and Transplantation,
  Leuven, Belgium.
  Promotor: Prof. Dominique Bullens
  Co-promotor: Prof. Lieven Dupont
- **Bachelor in Biomedical Laboratory Technology**, 2019 – 2020
  Graduated Cum Laude, University Colleges Leuven-Limburg (UCLL), Leuven, Belgium.
- **Master in Biomedical Sciences**, 2016 – 2018
  Graduated Cum Laude, KU Leuven, Leuven, Belgium
  Master thesis: 'Blood based biomarkers for the diagnosis and prognosis of amyotrophic lateral sclerosis'
  Promotor: Prof. Koen Poesen
- **Bachelor in Biomedical Sciences**, 2013 – 2016
  Graduated Cum Laude, KU Leuven, Leuven, Belgium
- **Sciences and Mathematics**, 2007 – 2013
  Heilig-Hartcollege, Heist-op-den-Berg, Belgium

Research experience

- **Doctoral Researcher**, 2018 – 2023
  'Study of airway inflammation as a result of external triggers inducing epithelial cell damage in exercise-induced bronchoconstriction'
  Allergy and Clinical Immunology Research Group, KU Leuven, Leuven, Belgium.
- **Master thesis**, 2017 – 2018
  'Blood based biomarkers for the diagnosis and prognosis of amyotrophic lateral sclerosis'
  Laboratory for Molecular Neurobiomarker Research, KU Leuven, Leuven, Belgium.
- **Laboratory internships in context of Master degree program**, 2016 – 2017
  Laboratory of Clinical and Experimental Endocrinology
  Laboratory of Pneumology
  Laboratory of Nuclear Medicine & Molecular Imaging