DISSERTATION FROM THE
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Julie Sørbø Stang

Why do athletes develop asthma?

Pathogenic mechanisms and asthma phenotypes

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Summary

Background: Asthma is reported frequently in endurance athletes, particularly among swimmers and cross-country skiers. However, the mechanisms of asthma development in athletes are not fully understood, and they seem to differ from the mechanisms reported in non-athletes. High-intensity endurance exercise accompanied by high ventilation rates ($\dot{V}E$) combined with unfavourable environmental exposures, such as exposure to chlorinated swimming pools or cold, dry air, is reported to induce epithelial damage in the airways. However, current evidence is not complete concerning the relationships between airway inflammation, systematic endurance exercise and bronchial hyperresponsiveness (BHR). Furthermore, the influence of other physiological adaptations to endurance exercise is not clear. In addition, sports asthma has been proposed as a specific phenotype of asthma, but this hypothesis has not yet been verified.

The physiological adaptations to endurance exercise include the autonomic nervous system, which mediates the contraction and relaxation of bronchial smooth muscle, with cholinergic-parasympathetic nerves stimulating bronchoconstriction. Parasympathetic activity is reported to be increased in endurance athletes and to correlate with maximal oxygen uptake ($\dot{V}O_{2max}$). As BHR denotes an increased bronchoconstrictor response to different stimuli (such as cold air, exercise or pharmacologic substances), increased parasympathetic activity in athletes could also increase the bronchomotor tone and the susceptibility to bronchospasm and thus BHR. In this regard, the measurement of parasympathetic activity in athletes with asthma may be of clinical value for better understanding the development of asthma in athletes. However, it remains unknown how different measurement procedures vary in terms of target organ.

Objectives: The present thesis aimed to investigate the pathogenic mechanisms of asthma in athletes, with an emphasis on the roles of the parasympathetic nervous system and airway inflammation in BHR, as well as asthma phenotypes. Swimmers and cross-country skiers were specifically targeted due to the high prevalence of asthma and BHR reported in the literature.

Material and methods: The present thesis is based on two separate studies, with the results presented in three papers. The first study had a cross-sectional design and included healthy and asthmatic swimmers (n=29) and cross-country skiers (n=28), as well as healthy non-athletes (n=30). The subjects made two visits to the laboratory, where measurements of parasympathetic activity by heart rate variability (HRV) and pupillometry were performed, and BHR to methacholine and airway inflammation were measured by assessing cells in induced sputum. The

primary aim was to assess the associations between parasympathetic activity (measured by HRV and pupillometry) and BHR to methacholine (Paper 1). A secondary aim of this study was to examine the presence of airway inflammation, as well as the relationship between airway inflammation and BHR (Paper 2). Twenty asthmatic athletes (10 swimmers and 10 cross-country skiers), 19 healthy athletes (10 swimmers and 9 cross-country skiers) and 24 healthy non-athletes were able to produce valid sputum samples and were included in the third paper. The second study was a cross-sectional study in which we examined the medical records of 150 elite athletes in Norway and Portugal and used latent class analyses to characterize asthma phenotypes based on clinical characteristics. We also evaluated the association between asthma phenotype and the type of sport practiced (paper 3).

Results: An association was found between BHR and HRV, but not between BHR and pupillometry (Paper 1). These associations were stronger in swimmers than in non-athletes, irrespective of asthma diagnosis. In addition, severe BHR was more frequent in swimmers than in cross-country skiers. Sputum inflammatory cells were not increased in either group, and no correlation with BHR was found. However, sputum interleukin-8 was increased in both healthy and asthmatic athletes in comparison to non-athletes (Paper 2). Two asthma phenotypes were identified in athletes: "atopic asthma" and "sports asthma." An increased risk of "sports asthma" was found among water sport athletes and winter sport athletes in comparison to land-based athletes and "summer athletes" (Paper 3).

Conclusions: Altogether, the results presented in the present thesis suggest that BHR, increased parasympathetic activity and exposure related to the type of sport practiced (training environment or type of training) contribute to the development of "sports asthma" – a distinct phenotype of asthma in athletes.

Sammendrag (Summary in Norwegian)

Bakgrunn: Astma er hyppig rapportert blant idrettsutøvere innen utholdenhetsidretter, og spesielt blant svømmere og langrennsløpere. Imidlertid er mekanismene for astma hos idrettsutøvere ikke fullstendig forstått, men ser ut til å avvike fra ikke-idrettsutøvere. Systematisk utholdenhetstrening med høy intensitet og tilsvarende høy ventilasjon ($\dot{V}_E$) kombinert med eksponering for ugunstig miljø, for eksempel fra klorholdige svømmebasseng eller kald og tørr luft, er faktorer som er foreslått å indusere epitelskade i luftveiene. Imidlertid er evidensen tilgjengelig idag ikke entydig når det kommer til sammenhengen mellom luftveisinflammasjon, systematisk utholdenhetstrening og bronkial hyperreaktivitet (BHR). I tillegg er det ikke klart om disse mekanismene er påvirket av andre fysiologiske tilpasninger til utholdenhetstrening. Videre har spesifikke fenotyper av idrettsastma blitt foreslått, men aldri testet.

De fysiologiske tilpasninger til utholdenhetstrening inkluderer det autonome nervesystemet, som regulerer kontraksjon og dilatasjon av bronkial glatt muskulatur hvor kolinerge-parasympatiske nerver stimuler til bronkokonstriksjon. Parasympatisk aktivitet er rapportert å være forhøyet hos idrettsutøvere innen utholdenhetsidretter, samt til å korrelere med maksimalt oksygenopptak ($\dot{V}O_{2max}$). Siden BHR karakteriseres av en økt bronkokonstriktorrespons til forskjellige stimuli, som for eksempel kald luft, fysisk aktivitet eller farmakologiske stimuli, kan økt parasympatisk aktivitet i teorien også disponere for økt bronkomotorisk tonus og videre til bronkospasme. I denne forbindelse, kan målingen av parasympatisk aktivitet hos idrettsutøvere med astma være av klinisk verdi for bedre å forstå utvikling av astma hos idrettsutøvere. Det er imidlertid ukjent hvordan forskjellige måleprosedyrer varierer når det gjelder målorgan.

Hensikt: Det overordnede målet med denne avhandlingen var å undersøke mekanismer for astma hos idrettsutøvere med hovedvekt på rollen til det parasympatiske nervesystemet og luftveisinflammasjon, deres rolle i sammenhengen med BHR, samt på fenotyper av astma. Svømmere og langrennsløpere ble spesielt fokuset på grunnet den høye forekomsten av astma og BHR som er rapportert i litteraturen blant disse typene idrettsutøvere.

Materiale og metode: Denne avhandlingen er basert på to studier og resultatene er presentert i tre artikler. Den første studien var en tverrsnittsstudie hvor friske og astmatiske svømmere (n=29) og langrennsløpere (n=28), samt friske ikke-utøvere (n=30) ble inkludert. Deltakerne møtte opp på laboratoriet på to ulike dager hvor målinger av parasympatiske aktivitet ved (hjerterytmevariabilitet, HRV) og pupillometri, BHR til metakolin og luftveisinflammasjon ved indusert sputum ble utført. Hovedmålet var å vurdere sammenhengen mellom parasympatisk

aktivitet og BHR til metakolin (Artikkel 1). Et sekundært mål var å undersøke grad av luftveisinflammasjon, så vel som forholdet mellom luftveisinflammasjon og BHR blant idrettsutøverene (Artikkel 2). Tjue astmatiske idrettsutøvere (10 svømmere og 10 langrennsløpere), 19 friske idrettsutøvere (10 svømmere og 9 langrennsløpere) og 24 ikke-utøvere produserte gyldige sputumprøver og ble inkludert i Artikkel 2. Den andre studien var en tverrsnitts studie der vi undersøkte journalene til 150 toppidrettsutøvere og brukte siden en analyse til å klassifisere ulike fenotyper av astma basert på kliniske karakteristika. Vi studerte i tillegg sammenhengen mellom risikoen for astma med hvilken type idrett utøveren konkurrerte i. Resultatene er presentert i Artikkel 3.

Resultater: En assosiasjon mellom BHR til metakolin og HRV, men ikke pupillometri, ble funnet (Artikkel 1). Sammenhengen mellom BHR og parasympatisk aktivitet var sterkere blant svømmere sammenlignet med ikke-idrettsutøvere, og var uavhengig av astmadiagnose. I tillegg var forekomsten av alvorlig BHR høyere hos svømmerne enn hos langrennsløperne. Inflammasjonsceller i indusert sputum var ikke økt hos noen av gruppene, og ingen sammenheng med BHR ble funnet. Imidlertid hadde både astmatiske og friske idrettsutøvere forhøyete verdier av interlaukin-8 i sputum, sammenlignet med kontroller (Artikkel 2). I den siste studien (Artikkel 3) avdekket vi to ulike fenotyper av astma blant idrettsutøvere; en "atopisk astma" og en "idrettsastma". Økt risiko for "idrettsastma" ble funnet blant idrettsutøvere innen vannsport og vintersport i forhold til landbaserte idrettsutøvere.

Konklusjon: Hovedresultatene fra disse studiene tyder på at BHR, økt parasympatiske aktivitet og spesifikke påvirkninger fra utøverens idrettsdeltagelse (treningsmiljø og/eller type trening) er involvert i mekanismene for "idrettsastma" - en egen fenotype av astma blant idrettsutøvere.

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Oslo, 7 October 2017

Julie S. Stang

List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. Stang J, Stensrud T, Mowinckel P, Carlsen KH. Parasympathetic activity and bronchial hyperresponsiveness in athletes. Med Sci Sports Exerc. 2016;48(11):2100-2107.

II. Stang J, Sikkeland, LIB, Tufvesson E, Holm A, Stensrud T, Carlsen KH. The role of airway inflammation and bronchial hyperresponsiveness in athlete's asthma. *Manuscript accepted for publication in Med Sci Sports Exerc.*

III. Couto M, Stang J, Horta L, Stensrud T, Severo M, Mowinckel P, Moreira A, Carlsen KH. Two distinct phenotypes of asthma in elite athletes identified by latent class analysis. J Asthma. 2015;52(9):897-904.

Abbreviations

ACh: Acetylcholine

ACV: Average pupil constriction velocity

AMP: Pupil amplitude

ANOVA: Analysis of variance

ANS: Autonomic nervous system

CI: Confidence interval

CON: Percent pupil constriction

CVI: Cardiac vagal index

EIA: Exercise-induced asthma

EIB: Exercise-induced airway inflammation

FEF_{25-75}: Mean forced expiratory flow between 25-75% of the forced expiratory volume

FEF_{50}: Forced expiratory flow at 50% of the forced expiratory volume

FE_{NO}: Fraction of exhaled nitric oxide

FEV_1: Forced expiratory volume in one second

FEV_1/FVC: Ratio of forced expiratory volume in one second to forced vital capacity

FVC: Forced vital capacity

HR: Heart rate

HRV: Heart rate variability

LoA: Limits of agreement

MCV: Maximal pupil constriction velocity

PD_{20met}: Inhaled cumulative dose of methacholine causing a 20% reduction in FEV_1

PNS: Parasympathetic nervous system

SD: Standard deviation

SNS: Sympathetic nervous system

SPT: Skin prick test

$\dot{V}_E$: Minute ventilation

$\dot{V}O_{2max}$:Maximal oxygen uptake

4sET: Four-second exercise test

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Introduction

Asthma is the most common chronic condition among Olympic athletes (Fitch, 2012), and the prevalence of asthma and bronchial hyperresponsiveness (BHR) is higher in endurance athletes than in other athletes or in the general population (Carlsen et al., 2008). Frequently performed high-intensity endurance exercise is believed to contribute to the development of asthma and BHR through regularly repeated high ventilation rates ($\dot{V}_E$), which cause significant mechanical stress to the respiratory epithelium, as well as increased environmental exposure (Carlsen et al., 2008; Haahtela, Malmberg, & Moreira, 2008). However, non-consistent airway inflammatory features are observed in athletes (Bougault, Turmel, St-Laurent, Bertrand, & Boulet, 2009), and the role of airway inflammation in asthma in athletes is not clear.

Increased activity in the parasympathetic nervous system has been reported in endurance athletes (Aubert, Seps, & Beckers, 2003; Filipe, Falcão-Reis, Castro-Correia, & Barros, 2003), and parasympathetic activity correlates with cardiorespiratory fitness ($\dot{V}O_{2max}$) (Buchheit & Gindre, 2006; Goldsmith, Bigger, Jr., Bloomfield, & Steinman, 1997). The autonomic nervous system affects visceral body functions, including the parasympathetic branch of nervus vagus, which regulates the constriction of the bronchi. It has been suggested that the increased parasympathetic activity associated with systematic endurance training may predispose endurance athletes to increased bronchomotor tone and increase susceptibility to bronchospasm and BHR (Moreira, Delgado, & Carlsen, 2011; Knöpfli & Bar-Or, 1999). In addition, cooling of the airways during exercise may cause exercise-induced bronchoconstriction (EIB) via increased vagal efferent tone (McFadden, Jr. & Ingram, Jr., 1979). Different procedures have been used to measure activity in the autonomic nervous system in athletes, but it is not known how different measurement procedures vary in terms of target organ.

Clinical asthma characteristics in athletes often differ from those observed in non-athletes (Voutilainen, Malmberg, Vasankari, & Haahtela, 2013; Lund, Pedersen, Anderson, Sverrild, & Backer, 2009). Based on the currently available evidence, it seems plausible that the mechanisms of asthma differ between athletes and non-athletes and possibly between athletes from different sports disciplines (Helenius, Tikkanen, Sarna, & Haahtela, 1998; Haahtela et al., 2008). However, it is not clear if a specific phenotype of "sports asthma" exists. The present thesis is based on three papers with the shared objective to better understand the specific mechanisms of asthma in athletes, particularly the roles of the parasympathetic system and airway inflammation in BHR, as well as to test specific asthma phenotypes in athletes.

Theoretical background

Definitions

Asthma is a respiratory disease that can be referred to as an umbrella term due to the heterogeneity of the condition. This heterogeneity is reflected in the currently used definition of the Global Initiative of Asthma (GINA):

> *«Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation"*
>
> The Global Initiative of Asthma (GINA), 2017.

Bronchial hyperresponsiveness (BHR) is defined as increased sensitivity to a wide variety of airway-narrowing stimuli, such as cold air, exercise or pharmacologic substances (Cockcroft, 2010; Sterk & Bel, 1989). This hypersensitivity is accompanied by excessive degrees of airway narrowing (bronchoconstriction) and is one of the major pathophysiological characteristics of asthma (Bateman et al., 2008). Although BHR is a feature of asthma and a majority of asthmatics have BHR, this state is not exclusive to asthma and may also be present to a mild to moderate degree in healthy subjects (Cockcroft, 2010; Nja, Roksund, Svidal, Nystad, & Carlsen, 2000).

Exercise-induced bronchoconstriction (EIB) refers to the transient narrowing of the airways following vigorous exercise and is defined as a ≥12% reduction in lung function, measured as the forced expiratory volume in one second (FEV_1), after a standardized exercise test (Carlsen et al., 2008; European Respiratory Society, 1997).

Exercise-induced asthma (EIA) describes the symptoms and signs of asthma provoked by exercise.

Diagnosis of asthma in athletes

Official guidelines for the diagnosis and treatment of athlete's asthma are established (Carlsen et al., 2008). The diagnosis of asthma in athletes is clinical and based on a history of symptoms, a physical examination with signs indicating the presence of bronchial obstruction and spontaneous or bronchodilator-induced variability in lung function (Carlsen et al., 2008). However, the diagnosis of asthma may be challenging in athletes, specifically due to the variability and non-specificity of respiratory symptoms, most often in relation to physical exercise (Rundell et al., 2000; Dickinson, Whyte, McConnell, & Harries, 2006; Lund, Pedersen, Anderson, Sverrild,

& Backer, 2009). Therefore, it is an important criterion that the diagnosis of asthma in athletes is confirmed by objective clinical findings (Carlsen et al., 2008).

Measurement procedures for BHR

Non-specific BHR can be measured using indirect or direct standardized bronchial provocation challenges. Indirect tests, such as an exercise test, a eucapnic voluntary hyperpnoea (EVH) test or the inhalation of mannitol, act through inflammatory mechanisms, causing the release of mediators from basophilic and eosinophilic granulocytes, which results in smooth muscle contraction with subsequent bronchoconstriction (Carlsen, Hem, & Stensrud, 2011). A direct provocation test stimulates the bronchial smooth muscle and bronchial glands directly via a pharmacological stimulus (e.g., inhaled methacholine or histamine) (Pauwels, Joos, & Van der Straeten, 1988).

The mechanisms and mediators of direct and indirect stimuli reflect different underlying mechanisms in the airways, which may explain the sometimes limited associations reported between these types of challenges (Van, Pauwels, & Joos, 2005). Climatic conditions during exercise, such as temperature or the humidity of the surrounding air, influence the magnitude of the airway response to exercise (Stensrud, Berntsen, & Carlsen, 2006; Stensrud, Berntsen, & Carlsen, 2007). Rundell et al. (2000) found that 98% of elite winter sport athletes reporting EIB had a positive test, while 48% of athletes who did not report EIB had a positive exercise test, using real life competitive events as the provoking agents. Those authors concluded that without relevant provoking agents, such as a sport-specific exercise field test, one might risk false negative results when screening for EIB or BHR among athletes. However, Dickinson et al. (2006) reported that an EVH test is a more sensitive challenge in asymptomatic athletes than a sport-specific and laboratory-based exercise challenge. Furthermore, Stensrud, Mykland, Gabrielsen and Carlsen (2007) reported that a methacholine bronchial challenge was a more sensitive test than a sport-specific test in high-level cross-country skiers. Stadelmann and colleagues (2011) found that a methacholine bronchial challenge had higher sensitivity for respiratory symptoms in swimmers than an EVH test but that the two tests compared well.

Prevalence of asthma and BHR in athletes

Asthma is the most common chronic medical condition among Olympic athletes (Fitch, 2012; Fitch et al., 2008), and asthma is more prevalent among endurance athletes than the general population (Carlsen et al., 2008). The increased prevalence of asthma, asthma-like symptoms and BHR among elite athletes was first reported in cross-country skiers (Larsson et al., 1993; Heir &

Oseid, 1994). These studies was followed by reports of both increased EIA and BHR among participants on the 1998 American Olympic National team for winter sports (Wilber et al., 2000). Similar findings were also reported among both summer and winter Olympic athletes with regard to the use of anti-asthmatic drugs (Fitch, 2006).

The risk of developing asthma has been associated with the type of training (Helenius, Tikkanen, & Haahtela, 1997), and asthma prevalence in athletes varies among different types of sports (Fitch, 2012). A higher occurrence of asthma and BHR is reported in endurance athletes, particularly swimmers and cold-air athletes (Langdeau & Boulet, 2001; Fitch, 2012). However, the definition of asthma and the measurement procedure for BHR, such as an indirect vs. direct test, influence variations in asthma prevalence in the literature. In a group of elite Swedish cross-country skiers, the prevalence of either asthma symptoms or BHR to methacholine was as high as 80% (Larsson et al., 1993). In a Canadian study, evidence of asthma, defined as at least one positive objective bronchial provocation test in response to either methacholine or exercise, was shown in 69% of swimmers, 28% of cold-air athletes, including cross-country skiers and speed skaters, and 17% of non-athlete controls (Bougault et al., 2009).

Mechanisms of "sports asthma"

The mechanisms of EIB are related to the airway response to heating and humidifying large volumes of inhaled air during high-intensive exercise (Anderson & Daviskas, 2000). Unlike this acute response to exercise, which may also occur in non-athletes, evidence suggests that the risk of developing asthma in athletes is related to the long-term effect of systematic high-intensive exercise with high $\dot{V}_E$ rates (Carlsen, 2013) (Figure 1). Training intensity, environmental exposure and viral infections are reported to influence BHR and airway inflammation in elite cross-country skiers (Heir & Larsen, 1995; Karjalainen et al., 2000).

The first study that showed a negative effect of endurance exercise on the airways demonstrated that the BHR to histamine (PC_{20}) was increased in both healthy and asthmatic children after swimming, and exercise intensity, measured as increased serum lactate, correlated significantly with the increase in BHR (Carlsen, Oseid, Odden, & Mellbye, 1989). It was also demonstrated that athletes with EIB or BHR trained more hours per week than healthy athletes (Couillard, Bougault, Turmel, & Boulet, 2014). Over time, the frequent exercise performed by competitive endurance athletes may result in epithelial damage and increased mucosal inflammation in the airways, together with delayed epithelial repair due to the daily repetition of hours of exercise (Helenius et al., 1997). Furthermore, there is evidence from both questionnaires and objective tests that BHR and asthma increase with age in cross-country skiers (Stensrud, Mykland,

Gabrielsen, & Carlsen, 2007; Heir & Oseid, 1994). This finding suggests that accumulated years of sports participation increase the risk of asthma.

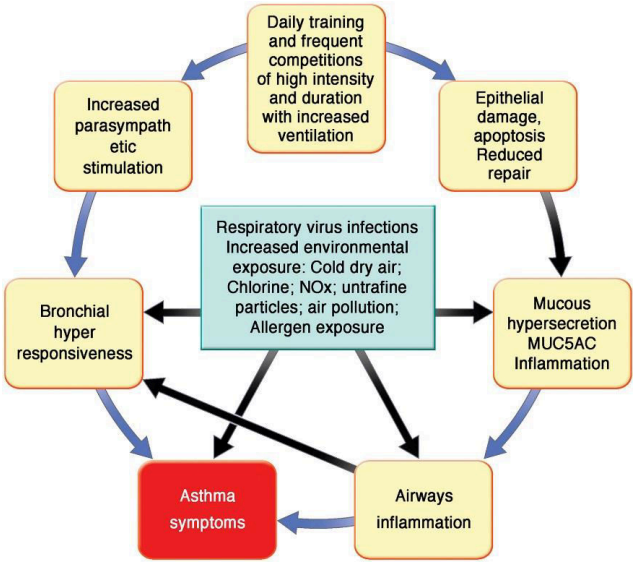


Figure 1 Overview of probable pathogenic mechanisms for the development of asthma in athletes. NOx, Nitrogen oxide. Reprinted from J Allergy Clin Immunol, 138/2, Carlsen KH, Lødrup Carlsen KC, Asthma and the Olympics, 409-10, Copyright (2016), with permission from Elsevier.

Airway inflammation and epithelial damage in athletes

Increased airway neutrophils and eosinophils are found in sputum from swimmers (Helenius, Rytila, Metso, Haahtela, Venge & Tikkanen, 1998; Bougault et al., 2009) and ice-hockey players (Lumme et al., 2003) in comparison to healthy non-athletes. However, lower levels of sputum eosinophils are found in swimmers and winter-sport athletes than in asthmatics (Bougault et al., 2009). Acute airway inflammatory responses to long-distance running (Bonsignore et al., 2001; Chimenti et al., 2010) have been shown after the examination of induced sputum. Bonsignore et al. (2001) showed that increased sputum neutrophils were present in non-asthmatic runners after a marathon run. However, contradictory findings are also reported, such as reports of the absence of signs of airway inflammation in athletes, despite the presence of BHR or asthma-like symptoms (Turmel, Bougault, & Boulet, 2012; Pedersen, Lund, Barnes, Kharitonov, & Backer, 2008; Martin, Lindley, Hargadon, Monteiro, & Pavord, 2012).

The airway epithelium is the first line of defence protecting the sensory nerves and smooth muscle from stimulation by inhaled irritants (Goldie et al., 1990). When the epithelial layer is damaged, the sensory nerves are exposed more directly, releasing neuropeptides that may induce bronchoconstriction. Bronchial biopsies of young cross-country skiers revealed increased airway

inflammation and epithelial damage after a competitive winter season in both asthmatic and non-asthmatic skiers (Sue-Chu et al., 1998; Karjalainen et al., 2000). Similar findings were reported in swimmers by Bougault et al. (2012). High V_E rates induce mechanical stress upon the airway wall. In addition to the release of airway inflammatory mediators, this stress may also damage the epithelial layer, which may result in airway remodelling over time (Bougault et al., 2012; Kippelen et al., 2012). Chimenti et al. (2010) found increased levels of sputum interleukin (IL)-8 and increased serum Club Cell protein 16 (CC16) in non-asthmatic runners after half-marathon runs. Furthermore, increased bronchial epithelial cells were found in sputum, but no change in sputum neutrophils was observed after a race. IL-8 is a chemokine and inflammatory mediator that has been shown to correlate with sputum neutrophils in healthy children (Kulkarni, Cooke, & Grigg, 2007) and adults with persistent, non-eosinophilic asthma (Gibson, Simpson, & Saltos, 2001). CC16 in plasma and urine has been reported as a marker of bronchial epithelial damage in athletes (Bolger et al., 2011; Tufvesson, Svensson, Ankerst, & Bjermer, 2013).

Environmental exposure

The airways of endurance athletes experience increased environmental exposure due to their high V_E during exercise. The inhalation of cold air, traffic pollution with particulate matter (diesel exhaust particles), nitrogen oxides (NO_x) and ozone (O_3), and organic chlorine by-products in the ambient air of indoor swimming pools may aggravate the development of exercise-induced airway inflammation and epithelial damage (Carlsen, 2013; Drobnic, Freixa, Casan, Sanchis, & Guardino, 1996). During a winter season, it has been shown that BHR to methacholine increases in elite cross-country skiers but not in control subjects (Heir & Oseid, 1994). Furthermore, airway inflammation and epithelial damage are found after strenuous exercise in cold weather in both humans (Sue-Chu et al., 1998; Karjalainen et al., 2000) and Alaskan sled dogs (Davis et al., 2002). This finding may explain why winter sport athletes, such as cross-country and biathlon skiers, as well as swimmers, are among the athletes with the highest prevalence of asthma, BHR and exercise-induced respiratory symptoms (Bougault, Turmel, & Boulet, 2010; Sue-Chu, Henriksen, & Bjermer, 1999).

Asthma phenotypes in athletes

Asthma is a heterogeneous disease (Aas, 1981) and studies have increasingly focused on the concept that asthma consists of multiple phenotypes or consistent groupings of characteristics, in both the adult (Fajt & Wenzel, 2015) and paediatric literature (Lodrup Carlsen et al., 2014). Asthma phenotypes may encompass different physiologic and pathologic characteristics. Therefore, the identification of asthma phenotypes will enable a better understanding of the

underlying mechanisms and may lead to more targeted and personalized approaches to asthma therapy.

In a review by Haahtela and colleagues (2008), the hypothesis that different asthma phenotypes occur in athletes was introduced. In this paper, two different clinical phenotypes were proposed, but those phenotypes have not been fully established. One phenotype is defined as a "classic asthma," and is characterized by an early onset (during childhood), with methacholine hyperresponsiveness, atopy and eosinophilic airway inflammation. The other phenotype has a later onset of symptoms (during sport careers) and is characterized by BHR to EVH and variable associations with atopic markers and eosinophilic airway inflammation. Interestingly, recent observations suggest a shift from the first phenotype to the latter in competitive endurance athletes (Carlsen, 2013). Different airway inflammatory features are observed in swimmers and cross-country skiers (Bougault et al., 2009), but it is not clear whether phenotypes are related to the type of sport or the training environment.

The autonomic nervous system

The autonomic nervous system (ANS) acts as an involuntary control system related primarily to visceral functions and regulates internal organs and glands. The ANS nerves are divided into sensory (afferent) and motor (efferent) subsystems and consist of two main divisions: the parasympathetic nervous system (PNS) and the sympathetic nervous system (SNS). These two divisions function through different pathways and act as complementary but oppositional systems (Hall & Guyton, 2006, pages 748-760). The PNS is responsible for stimulating activities that occur when the body is at rest, especially digestion but also the regulation of salivation, lacrimation (tears), urination and defecation. The SNS is responsible for stimulating activities associated with acute stress responses and physical activity (Table 1).

The ANS plays a primary role in airway calibre regulation through the innervation of the smooth muscle surrounding the bronchial tree, bronchial vessels and mucus glands (de Jongste, Jongejan, & Kerrebijin, 1991). The tonus of the airway smooth muscles is influenced by neurotransmitters, hormones and other mediators that work through either excitatory (agonist) or inhibitory (antagonist) effects in connection with specific receptors on the smooth muscle cells. The parasympathetic branch is responsible for the involuntary movements of bronchial smooth muscles and can induce either bronchoconstriction or bronchodilatation when activated or inhibited, respectively, as well as stimulating of respiratory mucus glands (Mazzone & Canning, 2002). It has also been described that acetylcholine is synthetized in the respiratory epithelium by cholineacetyltransferase and is released from epithelial cells (Wessler & Kirkpatrik, 2008).

Table 1 Effects of the parasympathetic and sympathetic nervous systems on various organs.

TARGET ORGAN/SYSTEM	PARASYMPATHETIC EFFECTS	SYMPATHETIC EFFECTS
HEART MUSCLE	Decreases rate of contraction	Increases rate and force of contraction
CORONARY BLOOD VESSELS	Causes vasoconstriction	Causes vasodilation
LUNGS	Causes bronchoconstriction	Causes bronchodilation, mildly constrict blood vessels
BLOOD VESSELS	Little or no effect	Increases blood pressure; causes vasoconstriction in abdominal viscera and skin; causes vasodilation in the skeletal muscles and heart during exercise
PUPILS	Constricts pupil	Dilates pupil
LIVER	No effect	Stimulates glucose release
CELLULAR METABOLISM	No effect	Increases metabolic rate
ADIPOSE TISSUE	No effect	Stimulates lipolysis
ADRENAL GLANDS	No effect	Stimulates secretion of epinephrine and norepinephrine
SWEAT GLANDS	No effect	Increases sweating
DIGESTIVE SYSTEM	Increases peristalsis and glandular secretion; relaxes sphincters	Decreases activity of glands and muscles; constricts sphincters
KIDNEY	No effect	Causes vasoconstriction; decreases urine formation

Adapted table 60-2 (page 754) from Hall & Guyton, 2006. Textbook of medical physiology (11 Ed.) Saunders/Elsevier, Philadelphia, PA.

Measurement procedures for parasympathetic activity

The ANS is interlinked with many physiological systems, and measurements of the responsiveness of the ANS in maintaining homeostasis may provide useful information about the functional adaptations of the body. The vagus nerve is the largest parasympathetic nerve innervating the oesophagus, trachea, heart, lungs, stomach, pancreas, liver and kidneys (Hall & Guyton, 2006, page 750). Thus, measurement protocols may target different organs to measure parasympathetic activity. In humans, the activity of the ANS has been evaluated using a variety of non-invasive test protocols, mostly regarding the cardiovascular system. Measurements of parasympathetic cardiac activity are used as a predicting factor for all-cause mortality in patients with cardiac diseases (Huikuri et al., 2000) and to monitor overtraining in athletes (Aubert et al., 2003). The methods described in the present thesis are limited to the most commonly used methods in research and clinical practice today.

Heart rate variability

The heart rhythm is defined by the rate of depolarization of cardiac pacemaker cells to contract the heart. The electrical and contractile activity of the heart is controlled primarily by both the

sympathetic and parasympathetic pathways working in parallel, but these pathways act through different structural pathways and transmitter systems (Levy, 1997). In a healthy heart with an intact ANS, continuous physiological variations of the sinus cycles occur, reflecting an autonomic balance and a normal variation in heart beats over time (Jose & Collison, 1970).

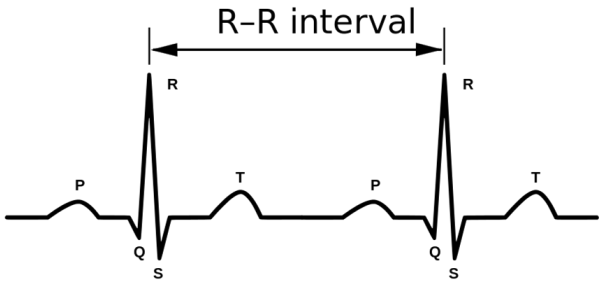


Figure 2 R-R intervals (ms) in a schematic diagram of a normal sinus rhythm for a human heart, as seen on an electrocardiogram (ECG). Source: Wikimedia commons.

Heart rate variability (HRV) describes the variations in time-intervals between consecutive heart beats and is calculated from the R-wave-to-R-wave (R-R interval) on an electrocardiogram (ECG) (Figure 2) or by a validated heart rate (HR) monitor (Task force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996; Achten & Jeukendrup, 2003; Vanderlei, Silva, Pastre, Azevedo, & Godoy, 2008; Gamelin, Berthoin, & Bosquet, 2006). Measurements of HRV can be analysed in the time or frequency domains (Task force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). Time domain analyses use statistical methods, such as standard deviations, to characterize the variation of R-R intervals, and frequency domain analysis describes the frequency at which the length of the R-R interval changes (Achten & Jeukendrup, 2003). Both methods have specific indices that are claimed to represent the activity of the different branches of the ANS separately, as well as their balance. Although a strong correlation between the time domain and frequency domain indices reflecting parasympathetic activity has been shown, inconsistent results have also been reported (Uusitalo, Tahvanainen, Uusitalo, & Rusko, 1996; Huikuri et al., 1999). HRV is a complex product of physical, mental and environmental factors and is influenced by circadian variation. Therefore, HRV measurements over 24 hours are often recommended, but short-term protocols have also been found to be useful, depending on the purpose of the study (Costa et al., 1994). During a breathing cycle, a natural variation in HR occurs, which is often referred to as respiratory sinus arrhythmia (RSA) (Grossman & Taylor, 2007). RSA can be quantified by HRV spectral or time domain analyses and is recognized as a measure of parasympathetic activity (Grossman & Taylor, 2007).

The balance of the autonomic function as a whole is closely linked to the activity of both branches of the ANS, making it challenging to measure the PNS or SNS separately. The processes that represent the functionality of the PNS are often isolated using pharmacological methods. The use of vagal blockade, which causes an immediate increase in heart rate, has been applied in various studies to assess cardiac parasympathetic activity. Similarly, physical activity induces an almost instantaneous vagal withdrawal to increase the HR, which is later followed by sympathetic stimulation to further increase the HR (Petro, Hollander, & Bouman, 1970; Tulppo, Mäkikallio, Seppänen, Airaksinen, & Huikuri, 1998; Fujii et al., 2000). Thus, at the onset of dynamic exercise, the initial HR transient is exclusively vagus-dependent. Based on this notion, Brazilian researchers introduced a short (4-second) exercise test to assess cardiac vagal activity (Araújo, Nobrega, & Castro, 1989). During this procedure, subjects must hold their breath to control for RSA. The test is called the 4-second exercise test (4sET) and has been validated via pharmacological blockade using atropine (Araújo, Nobrega, & Castro, 1992).

Pupillometry

Pupillometry is a measure of the autonomic balance of the parasympathetic and sympathetic systems upon pupil diameter regulation. The pupil diameter is controlled primarily by the smooth musculature in the iris, the m. dilatator pupillae and the m. constriction pupillae (Heller, Perry, Jewett, & Levine, 1990). The dilatator muscle is innervated by sympathetic nervous fibres, and the constrictor muscle is innervated by parasympathetic nerve fibres and inhibits the pupillary dilator muscles (Kaltsatou, Kouidi, Fotiou, & Deligiannis, 2011). Therefore, pupillometry allows for the independent evaluation of both branches of ANS activity, with the constrictive phase reflecting parasympathetic activity and the dilation phase reflecting sympathetic activity. During pupillometry, the eye is stimulated with a flash of light, and a rapid sequence of digital images captures the pupil diameter when it constricts and then redilates to its original size (Figure 3).

The following parasympathetic variables are acquired by pupillometry: percent pupil constriction, pupil amplitude (calculated from the difference between the initial and minimal pupil diameter) and the maximal and average constriction velocity (Figure 2). The sympathetic variables include the average dilation velocity (given in millimetres/second) and the total time taken by the pupil to recover 75% of the initial pupil size after it reaches the peak of constriction (given in seconds). The baseline pupil size, the latency for the onset of constriction, and the minimum pupil radius will reflect the sympathetic-parasympathetic balance (Fotiou et al., 2007; Filipe et al., 2003). Many parameters of pupillometry appear to be strongly age-related, such as the maximum constriction

acceleration and velocity (Fotiou et al., 2007). In addition, the pupil constriction amplitude and the percent constriction are dependent upon the baseline pupil size (Filipe et al., 2003).

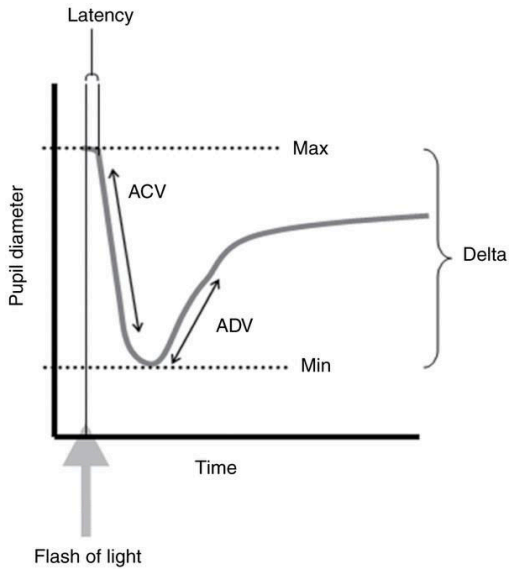


Figure 3 The pupil's reaction to light, as measured by pupillometry. ACV: average constriction velocity; ADV: average dilatation velocity. Reprinted with permission from Macmillan Publishers, Ltd.: [PEDIATRIC RESEARCH] Pallavi et al. 71(3);280-285, copyright 2012.

Pupillary measurements are acquired with portable, hand-held infrared pupilometers (Filipe et al., 2003) or set-ups that combine stationary digital high-speed video cameras with supplied software (Kaltsatou et al., 2011). The need for healthy eyes that are free of any ophthalmological disorders is a requirement of pupillometry. In general, systemic medications with known central nervous system effects and neurological illness are other factors that may influence this type of ANS assessment.

Parasympathetic activity and asthma

Decades ago, increased parasympathetic activity in the airways was proposed to contribute to BHR in asthmatic subjects, as the bronchoconstrictor effect of a variety of cholinergic stimulants in the airways was abolished after blocking cholinergic efferent pathways with an intravenous injection of atropine sulphate (Simonsson, Jacobs, & Nadel, 1967; Boushey, 1984). In addition, cooling of the airways during exercise may cause EIB through increased vagal efferent tone (McFadden, Jr. & Ingram, Jr., 1979). Increased vagal cardiac tone, as measured by HRV, is found in asthmatics and suggests that increased parasympathetic bronchial activity may influence the pathogenesis of asthma, reflected by a parallel change in the vagal control of the heart

(Kallenbach et al., 1985; Sturani, Sturani, & Tosi, 1985). Knöpfli and co-workers found an association between the parasympathetic stimulation of the heart and the bronchodilating effect of inhaled ipratropium bromide in cross-country runners exercising at -5°C (Knöpfli & Bar-Or, 1999) and in children with EIB (Knöpfli, Bar-Or, & Araújo, 2005). Further, subjects with BHR exhibited increased parasympathetic tone, as measured by HRV, after a methacholine bronchial challenge in comparison to subjects without BHR (Pichon, de Bisschop, Diaz, & Denjean, 2005).

Parasympathetic activity in endurance athletes

Increased parasympathetic activity, as measured with both HRV and pupillometry, has been shown in endurance athletes in comparison to non-athletes (Filipe et al., 2003; Shin, Minamitani, Onishi, Yamazaki, & Lee, 1997). Parasympathetic activity, as measured by HRV, has been shown to increase after an endurance exercise intervention (De Meersman, 1992), and a correlation between HRV and cardiorespiratory fitness ($\dot{V}O_{2max}$) was reported (Buchheit & Gindre, 2006; Goldsmith et al., 1997; Filipe et al., 2003; Knöpfli & Bar-Or, 1999). These findings suggest a clear effect of endurance exercise upon the ANS and explain the pronounced resting sinus bradycardia that has been found in endurance-trained subjects (Jensen-Urstad, Saltin, Ericson, Storck, & Jensen-Urstad, 1997).

HRV monitoring is used by elite athletes to evaluate the degree of restitution and to evaluate the physiological effects of different training programs (Aubert et al., 2003). Reduced parasympathetic HRV indices are associated with overtraining (Uusitalo, Uusitalo, & Rusko, 2000; Buchheit, Simon, Piquard, Ehrhart, & Brandenberger, 2004), and decreased parasympathetic HRV indices have been reported the night after strenuous exercise activities, such as a marathon run (Hynynen, Vesterinen, Rusko, & Nummela, 2010) or a 75 km ski-race (Hautala et al., 2001).

Research gap

An increased risk of asthma and increased parasympathetic activity have both been recognized in elite endurance athletes. As the cholinergic-parasympathetic nerves stimulate bronchoconstriction, it seems likely that increased parasympathetic activity could also predispose athletes to increased bronchomotor tone and susceptibility to bronchospasm. In fact, an association between diminished sweat secretion, tearing rates and salivary flow rates and methacholine bronchial responsiveness was found in healthy athletes, indicating an autonomic dysfunction (Park, Stafford, & Lockette, 2008). It has been suggested that autonomic dysfunction is related to the development of BHR and asthma in athletes (Moreira et al., 2011; Carlsen, 2013)

and that the reported variations in asthma prevalence between different types of athletes (i.e., endurance athletes) may be attributed to differential parasympathetic stimulation (Knöpfli & Bar-Or, 1999). However, whether increased parasympathetic activity is involved in the pathological mechanisms related to "sports asthma" remains to be clarified.

HRV typically reveals physiological differences between athletes and non-athletes related to the cardiovascular system. Pupillometry has also been shown to differ between endurance-trained athletes and non-athletes, yet the relationships between parasympathetic activity in the lungs, the heart and the pupils are uncertain. Parasympathetic bronchial tone was previously measured via cholinergic blockade, such as by using atropine (Deal, McFadden, Ingram, & Jaeger, 1978; Boushey, 1984; Araújo, Nobrega, & Castro, 1992) or ipratropium bromide (Knöpfli & Bar-Or, 1999), or via measurements of airway resistance (Horvath, Argay, Herjavecz, & Kollai, 1995).

The current evidence is conflicting regarding the role of airway inflammation in the mechanisms of asthma in athletes. Different inflammatory patterns in asthma are shown by induced sputum (Gibson et al., 2001), and signs of airway inflammation are often found after exercise, but not always at baseline, which may suggest that the acute inflammatory response to exercise is reversible. In a 5-year follow-up study, Helenius et al. (2002) showed an improvement of BHR in swimmers who had ended their sporting careers. However, more evidence is needed to establish whether BHR, airway inflammation, or epithelial damage persists beyond intensive periods of endurance exercise and competitions.

Asthma is defined as a heterogeneous condition, and different phenotypes are described in non-athletes. The characteristics of asthma in athletes may differ from those observed in the general population. For instance, the relationship between patterns of asthma-like symptoms and objective measurements is poorly defined in athlete's asthma. Some athletes appear to aggravate existing asthma through their exercise regimes, while others develop the disease. Furthermore, the differences among asthma mechanisms and the influence of the type of sport or the training environment are not fully accounted for. A review paper suggested that there are two phenotypes of asthma in athletes (Haahtela et al., 2008), but this statement has not been confirmed.

Objective and aims of the thesis

The main objective of the present thesis is to investigate the mechanisms of athlete's asthma, with a particular emphasis on the relationships between the parasympathetic nervous system and BHR, airway inflammation and asthma phenotypes.

More specifically, the main aims were as follows:

1. To examine the relationship between BHR and parasympathetic activity in competitive swimmers and cross-country skiers, as well as in healthy non-athletes, and whether this association is influenced by the target organ of parasympathetic activity measurement (Paper I).
2. To determine if parasympathetic activity is increased in athletes with asthma or BHR in comparison to healthy athletes and non-athletes (Paper I).
3. To compare airway inflammation and BHR between asthmatic and non-asthmatic swimmers and cross-country skiers, as well as with non-athletes, and to assess the relationship between airway inflammation and BHR (Paper II).
4. To assess asthma phenotypes in elite athletes and investigate their possible association with the type of sport practiced (Paper III).

Materials and methods

Study design and subjects

The present thesis includes results from two separate studies, which were both carried out at the Norwegian School of Sport Sciences (NSSS) in Oslo, Norway. Study II were conducted in collaboration with the University of Porto, Portugal.

Study I: Competitive cross-country skiers and swimmers, as well as healthy non-athletes, were included in this cross-sectional study. The primary aim was to assess the association between BHR, as measured by a methacholine bronchial challenge, and parasympathetic activity, as measured by pupillometry and 4sET. The secondary aims were to compare parasympathetic activity, BHR and airway inflammation between healthy and asthmatic cross-country skiers, swimmers and non-athletes. The inclusion criteria for athletes were to compete on a high-national or international level and to train >10 hours per week. The inclusion criteria for non-athletes were to train <5 hours per week and to not have asthma. The athletes were grouped based on whether they had current asthma. Current asthma was defined as a doctor's diagnosis of asthma in combination with current use of anti-asthmatic medications and/or current BHR (defined as an inhaled cumulative dose of methacholine causing a 20% reduction in FEV_1 [PD_{20met}] ≤8 μmol). The study included 28 cross-country skiers (♂18/♀10), 29 swimmers (♂17/♀12) and 30 non-athletes (♂14/♀16), aged 16-40 years. Fourteen swimmers (48%) and 16 cross-country skiers (57%) met the criteria for current asthma.

All subjects attended the laboratory at the NSSS on two separate days. On the first day, BHR was measured by a methacholine bronchial challenge. Airway inflammation was assessed based on induced sputum on day two. Measurements of parasympathetic activity by pupillometry and 4sET were performed on both days (paper I). Influence from potential circadian variations in parasympathetic activity were minimized by scheduling all visits at the same time of day. Twenty asthmatic athletes (10 swimmers and 10 cross-country skiers), 19 healthy athletes (10 swimmers and 9 cross-country skiers) and 24 non-athletes were able to produce valid sputum samples and were included in paper II.

Study II: In this cross-sectional study, we analysed the clinical characteristics of elite athletes with asthma. Data were retrieved from medical records kept in databases from the study "Asthma and allergy in Olympians," which included Norwegian participants in the 2008 Beijing and 2010 Vancouver Olympic Games, as well as registries from the Portuguese Anti-Doping Authority and the Portuguese database of Olympic athletes. We included all files for which information were

available concerning respiratory symptoms, lung function, airway inflammation by fractional exhaled nitric oxide (FE_{NO}), BHR and allergic sensitization. Healthy athletes and those with conditions other than asthma were excluded. A total of 324 files had complete information, including informed consent for data use. Of these files, 150 athletes (59 Norwegian and 91 Portuguese) fulfilled the criteria for asthma and were included in the study.

Subject restrictions and preparation

All subjects had been free from any respiratory disease for the three weeks before testing. If the subjects became ill between testing days (study I), both days were repeated after >3 weeks. On the day of the visit, the subjects were asked to refrain from exercise, and the intake of any food or drink containing caffeine, nitrate or other substances that may influence the tests was restricted (See Appendix III). Anti-asthmatic medication was withheld before testing (Miller et al., 2005). Inhaled short-acting β_2-agonists were withheld for 8 hours before testing; inhaled long-acting β_2-agonists, theophylline and leukotriene antagonists were withheld for 72 hours before testing; antihistamines were withheld for 7 days before testing; and orally administered glucocorticosteroids were withheld for one month before testing. Inhaled corticosteroids were not to be used on the day of testing.

Measurement procedures

Lung function

Lung function was measured by maximal expiratory flow-volume loops using a MasterScreen Pneumo spirometer (CareFusion, Höchberg, Germany [previously: Jaeger GmbH, Würzburg, Germany]) according to current guidelines (Miller et al., 2005) (Papers I & II). The predicted values used are according to Quanjer and co-workers (2012). The following variables were recorded: forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), and forced expiratory flow at 50% (FEF_{50}) or 25-75% of FVC (FEF_{25-75}).

Methacholine bronchial challenge

A methacholine bronchial challenge was performed via tidal breathing using an inspiration-triggered Aerosol Provocation System (APS) Jäger (Würzburg, Germany) (Papers I & II). The nebuliser output was controlled and calibrated before the start of the study and on a weekly basis during the study period. Lung function was measured before and after inhaling nebulised isotonic saline (0.9%). Then, the subjects inhaled doubling doses of methacholine chloride (32 $mg \cdot mL^{-1} \cdot$) from a starting dose of 0.51 μmol (0.1 mg) until a reduction in FEV_1 of 20% or the maximal dose of 24.48 μmol methacholine (4.8 mg) was reached. A positive response to methacholine was

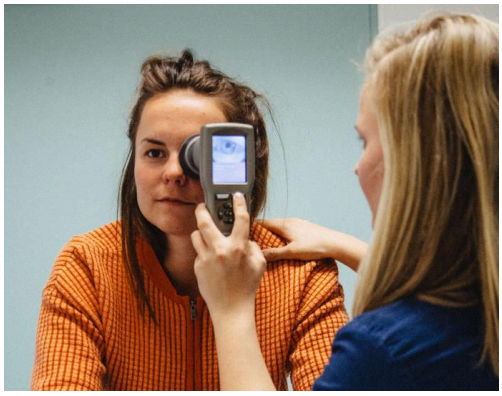
defined as a 20% reduction in FEV_1 from the reference value after saline inhalation. The methacholine provocation dose causing a 20% decrease in FEV_1 was calculated by linear interpolation on the dose-response curve and recorded as PD_{20met}. Clinically significant BHR was defined as $PD_{20met} \leq 8$ μmol. A stricter cut-off value of 2 μmol was also applied, similar to the cut-off for approval of the use of asthma medication in athletes that was previously used by the World Anti-Doping Agency (WADA) (WADA, 2009; Cockroft, 2010). All subjects received salbutamol inhalation (0.1 mg·mL^{-1}·10 kg body mass^{-1}) by nebulisation to reverse bronchial obstruction after the methacholine provocation.

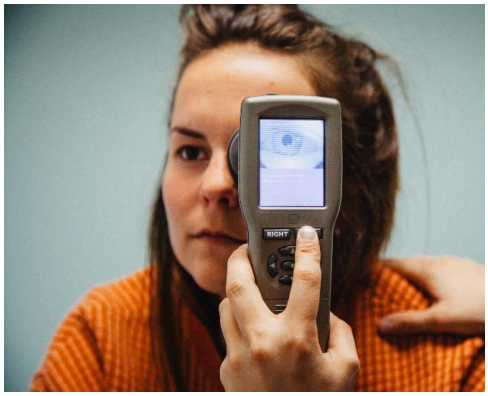
Induced sputum

Induced sputum was collected and processed as described by Alexis, Soukup, Ghio, & Becker (2000) (Paper II). All subjects were pre-treated with inhaled salbutamol (0.1 mg·ml^{-1}·10 kg body mass^{-1}) mixed in 1 ml of isotonic NaCl and delivered through a Sidestream nebulizing chamber (Respironics Respiratory Ltd, Chichester, UK) connected to a CR60 compressor (Medic-Aid Ltd, West Sussex, UK) at a flow rate of >6 L·min^{-1}. The subjects inhaled 3% (w/V), 4% and 5% hypertonic saline for 7 min via an ultrasonic nebulizer (DeVilbiss Healthcare Ltd., West Midlands, UK). After each inhalation, the subjects were asked to blow their nose, rinse their mouth, and perform a chesty-type cough. Expectorate was collected into a sterile container, and lung function tests were repeated. Sputum was processed within 2 hours after induction. Mucus plugs was separated from saliva, weighed and dissolved in phosphate-buffered saline (PBS, Dulbecco's PBS Invitrogen, Burlington, ON, Canada) containing 0.1% (w/V) dithiothreitol (DTT, Sigma, St. Louis, MO). The sample was mixed for 15 minutes, washed with PBS, filtered through a 48-μm pore mesh filter (Sintab, Oxie, Sweden) and centrifuged. The supernatants were frozen at -80°C. Total cell counts and cell viability were determined with a Bürker chamber using the trypan blue (0.4%) (Sigma) exclusion method. Calculations of cell differentiation were performed on blinded cyto-centrifuged preparations stained with Diff-Quick (Merz-Dade, Dudingen, Switzerland) and expressed as a percentage of the total. At least 400 cells/slide were counted by two investigators. The sputum sample was considered to be adequate if the sample was contaminated by <50% squamous epithelial cells and/or if the sample had >50% viability. Lung function was measured by maximal expiratory flow-volume loops before and 15 minutes after pre-treatment and each bout of saline inhalation.

Pupillometry

Pupillometry was assessed with the portable infrared PLR-200™ Pupillometer (NeurOptics Inc, CA, USA), which stimulated the eye with a light flash (180 nm peak wave light) and then captured a rapid sequence of digital images to measure the pupil diameter (Paper I). The subjects spent 15 minutes in a semi-dark room to adapt to low lighting levels before measurement. The subjects were then instructed to focus on a small target object with the eye that was not being tested, keeping the head straight and both eyes wide open during measurement (Picture 1 and 2). One pupil light response curve for each eye was recorded for each subject, starting with the left eye, with the mean values of both eyes used for further analyses. The following parameters were collected: the diameter of the pupil before and just at the peak of constriction (given in millimetres), the percent of constriction, the time of onset of constriction (given in seconds), and the average and maximum constriction velocity (given in millimetres/second). The pupil amplitude (mm) was calculated by subtracting the minimal diameter (at the peak of constriction) from the initial pupil diameter. Pupillometry was performed on two separate days, and the mean values were used for analyses.





Picture 1 and 2 Pupillometry measurement.

Four-second exercise test

The four-second exercise test (4sET) was performed on a cycle ergometer (Araújo et al., 1989) (Paper I). After R-R interval stabilization at rest, four verbal commands were given, in the following sequence: (0 s) take a deep inspiration, (4 s) cycle as fast as possible, (8 s) suddenly stop cycling, and (12 s) perform expiration. No load was applied to the cycle ergometer so the subjects could easily start to pedal as fast as possible, from the fourth to the eighth second of their maximal inspiratory apnoea. We measured R-R intervals with heart rate monitors from Polar

Electro® (OY, Kempele, Finland), which have been shown to be comparable to ECG (Nunan et al., 2009). The ratio between the longest R-R interval before exercise and the shortest R-R interval during the four-second cycling exercise was calculated as the cardiac vagal index (CVI) after manual identification (Figure 4). Two 4sET manoeuvres were performed, and the highest CVI value was used for further analyses (Araújo, Ricardo, & Almeida, 2003). The 4sET was performed on two separate days, and the mean values were used for analyses.

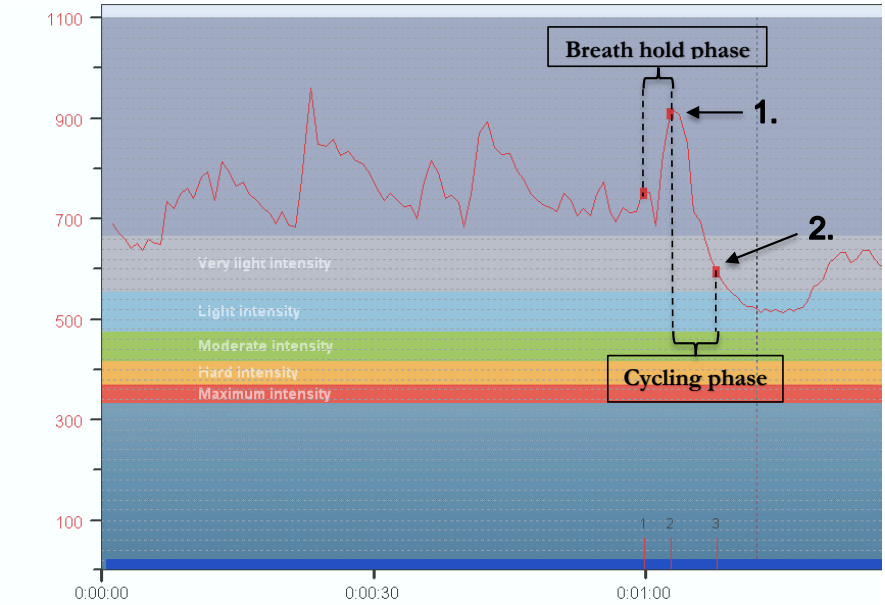


Figure 4 Measurements of R-R intervals (y-axis), given in milliseconds, during the four-second exercise test (4sET) in one subjects. Time (x-axis) is given in minutes and seconds. The test begins with a 4-second breath hold phase, followed by a 4-second cycling phase. The longest (point no. 1) and the shortest (point no. 2) R-R intervals, which were usually the last R-R intervals during the breath hold phase and the cycling phase, respectively, are identified. The ratio of these two values is used to define the cardiac vagal index (CVI).

Exhaled nitric oxide

Fractional exhaled nitric oxide (FE_{NO}) was measured using the Eco Medics CLD 88 sp Exhalyzer (Eco Medics AG, 8635 Duerten, Switzerland) with the single-breath technique, according to American Thoracic Society (ATS) and European Respiratory Society (ERS) guidelines (2005) (Papers I & II). The subjects inhaled NO-free air to total lung capacity and exhaled with a standardized flow of 50 mL/s and a target pressure of 20 cm H_2O. The mean values of three measurements with a <10% difference were used in the analysis.

Skin prick test

An allergy skin prick test (SPT) was carried out with extracts of 10 common allergens (ALK-Abelló as, Hørsholm, Denmark): dog, cat and horse dander, birch, timothy and mug wort

pollens, mould (*Cladosporium herbarium*), house dust mites (*Dermatophagoides pternoyssinus*), cow's milk and hen's egg white (Papers I & II). A subject was classified as allergic sensitized if at least one allergen caused a weal diameter of ≥3 mm greater than the negative control, in the presence of a negative saline control and a positive histamine control (Bousquet et al., 2012).

Questionnaire

A modified $AQUA_{2008}$ questionnaire, which was developed and validated for the assessment of asthma, allergy and other respiratory symptoms in athletes (Bonini et al., 2009) was administered to record past or present history of asthma, allergy and exercise-induced asthma-like symptoms (Papers I & II). Medical data collected included the presence of respiratory symptoms, current use of asthma medications and the presence of rhinitis or other allergic disease (conjunctivitis, urticaria, eczema, and anaphylaxis and allergies to drugs, food and venom). See Appendix II.

Definitions

Asthma diagnosis: to be established by a medical doctor according to the criteria set by the European Respiratory Society (ERS) and the European Academy of Allergy and Clinical Immunology (EAACI) to document asthma in athletes, including objective evidence of either reversibility after bronchodilator administration or BHR after a bronchial provocation challenge (Carlsen et al., 2008).

Current asthma: a diagnosis of asthma (as described above) in combination with current use of anti-asthmatic medication or current BHR, confirmed by a methacholine bronchial challenge.

Bronchial hyperresponsiveness: PD_{20met} ≤2 μmol (severe BHR) and PD_{20met} ≤8 μmol (clinical BHR), as measured by a methacholine bronchial challenge.

Parasympathetic activity was measured in two target organs:

1. In the heart, based on heart rate variability (HRV) during a four-second cycling exercise test (4sET) and calculated as the cardiac vagal index (CVI).
2. In the pupil, based on pupillometry and reflected in the parameters of the constriction phase of the pupil's reaction to a light stimulus: percent constriction (CON), amplitude (AMP) (mm), and maximal (MCV) and average constriction velocities (ACV) (mm/s).

The demographic data obtained included age, gender, height, weight and type of sport practiced. In study II, the type of sport was classified according to environmental training conditions. Water sports included swimming and water polo. Winter sports included cross-country and biathlon skiing, skeleton, alpine skiing and ski cross. Other sports included speed skating, curling,

equestrian, taekwondo, auto-racing, billiards, paragliding, rugby, tennis, roller hockey, kickboxing, fencing, basketball and golf.

Statistical analyses

Demographic data are presented as means with standard deviations (SD) or 95% confidence intervals (CI). The results are presented as means with 95% CI, unless otherwise stated. In the case of a skewed distribution, medians with interquartile ranges (IQR) were used. Categorical data are presented as counts with percentages, unless otherwise stated. Correlations were calculated using Spearman's rank order correlation (ρ) or Pearson's correlation coefficient, (r_p) where applicable. Group mean differences for two independent samples were assessed using the two-tailed Student's t-test for normally distributed continuous variables and the Mann-Whitney test for data with a skewed distribution. Analysis of variance (ANOVA) for three or more group comparisons was used, after tests for normality, with post hoc tests (Tukey's multiple comparisons technique) applied to determine within-group differences. Pearson's Chi square test (χ^2) or Fisher's exact test was used for categorical variables. All p-values below 0.05 (5%) were considered to be significant. Subjects who did not achieve a significant fall in FEV_1 in the methacholine bronchial challenge were assigned the maximal PD_{20met} value of 25 μmol, whereas subjects with a positive PD_{20met} fall on the first dose (<0.1 μmol) were assigned a value of 0.1 μmol.

In **paper I,** robust regression analyses were used to assess the associations between PD_{20met} with pupillometry and 4sET parameters, respectively, due to the non-normal distribution of residuals. Statistical analyses were calculated using Statistical Package for Social Sciences 21.0 (SPSS, Chicago, IL, USA), and SAS 9.4 (SAS Institute Inc., North Carolina, USA). The figures were generated using GraphPad Prism 6.0 (Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com).

In paper **II,** the three groups were compared using Kruskal-Wallis tests after tests for normality on continuous data. Chi square tests (χ^2) were used to assess group differences for categorical variables. The Mann-Whitney U Test for independent samples was used to compare athletes with non-athletes. Statistical analyses were performed using IBM SPSS Statistics 21.0 (SPSS Inc., Chicago, IL, USA). GraphPad Prism 6.0 (Windows, GraphPad Software, San Diego California USA, www.graphpad.com) was used to generate figures.

In **paper III**, asthma phenotypes in athletes were distinguished based on latent class analysis (LCA) to uncover distinct group patterns or case subtypes (latent classes) based on multivariate

categorical data. The analysis is based on the assumption that within latent classes, each variable is statistically independent of every other variable. The nine variables that were included in the LCA were selected based on the assumption of their clinical relevance for asthma definition (Table 2). The number of latent classes was defined according to Bayesian Information Criterion (BIC). Starting from one single class and increasing one class at each step, the best solution was identified when the increase in the number of classes did not lead to a decreased BIC. The association between asthma phenotype and type of sport (water or winter sport) was analysed using regression analysis to predict the odds of having a specific asthma pattern (phenotype), with "other sports" as a reference. Statistical analyses were calculated using Statistical Package for Social Sciences 20.0 (SPSS, Chicago, IL, USA), except for LCA models, which were fitted using MPlus (V.5.2; Muthen & Muthen, Los Angeles, CA).

Table 2 Definitions of the nine asthma-defining variables used in the Latent Class Analysis (LCA)

	Variable	Definition
1.	Airflow obstruction	FEV_1/FVC <0.70
2.	Airway hyperresponsiveness[1]	A fall in FEV_1 of ≥10% from baseline with exercise or EVH OR a fall in FEV_1 of ≥20% from baseline with inhaled methacholine: PD_{20} ≤400 μg (cumulative dose) in those not using ICS or PD_{20} ≤1600 μg (cumulative dose) in those using ICS for at least one month*.
3.	Allergic sensitization	The presence of ≥1 positive skin prick tests or the presence of a positive specific IgE (≥0.35kU/L) for ≥1 common allergen.
4.	Any other allergic disease	Positive answer to any of the following questions[2]: "Did any doctor diagnose you with an allergic disease?" "Do you frequently have red eyes with tearing and itching?" "Have you ever had severe allergic or anaphylactic reactions?" "Have you ever had an allergic reaction to foods?" "Have you ever had an allergic reaction to drugs?"
5.	Asthma treatment	Current or recent treatment with ICS and/or β_2-agonists
6.	Eosinophilic inflammation	The presence of FE_{NO} levels above 25 ppb
7.	Respiratory symptoms	Self-reported recurrent breathlessness, cough, wheeze, chest tightness and/or phlegm prodution[2]
8.	Reversibility	Increase of >12% and 200 mL in FEV_1
9.	Rhinitis	Positive answer to the following questions: "Did any doctor diagnose you with an allergic disease?" AND "rhinitis" OR "Do you frequently sneeze or have a running, itchy nose (apart from colds)?"

[1]According to the International Olympic Committee (IOC) Medical Commission; [2]From the AQUA questionnaire. FEV_1: forced expiratory volume in the first second; FVC: forced vital capacity; EVH: eucapnic voluntary hyperpnoea; ICS: inhaled corticosteroids; PD_{20}: provocative dose of methacholine causing a 20% decrease in FEV_1; FE_{NO}: fractional exhaled nitric oxide; IgE: Immunoglobulin E. *Only the most common definitions are included. Simplified from Couto et al. J Asthma, 2015.

Statistical power

Power calculations were performed in study I as outlined below.

The sample size determination and power assessment were based upon the distribution (SD) of parasympathetic activity parameters from a pilot study. With the reflex amplitude (mm) of pupillometry as the main outcome variable, an overall mean (SD) of 2.20 (0.30) was found. To achieve a power of 80% with a significance level of 0.05, thirty participants in each group would detect a difference of at least 0.18 between the control group and the athlete groups, and a difference of at least 0.06 between the athlete groups.

Ethical considerations

The present studies were reviewed by the Regional Committee for Medical and Health Research Ethics (REC) (See appendix I). Study I was approved by REC (2013/167) and registered in the Norwegian Bio-Bank registry at Oslo University Hospital. Study II was approved by REC (S-07468a), as well as the São João Hospital Centre in Porto, Portugal (174/12). All participants included in the present studies volunteered for participation and signed a written informed consent form, according to the Helsinki Declaration. The parents or guardians of subjects under the age of 18 years gave their written consent, in addition to the subjects themselves.

Results

Associations of BHR with parasympathetic activity (Paper I)

Twenty-eight cross-country skiers (♂18/♀10), 29 swimmers (♂17/♀12), and 30 healthy non-athletes (♂14/♀16) completed both data collection visits in study I. Fourteen swimmers (48%) and 26 cross-country skiers (59%) met the criteria set for current asthma.

Table 3 Associations (β coefficients with 95% confidence intervals [CI]) of bronchial hyperresponsiveness (BHR) to methacholine (PD_{20met}, dependent variable) with parasympathetic activity variables from pupillometry and heart rate variability (HRV) (independent variables) in competitive swimmers (n=29), cross-country skiers (n=28) and non-athletes (n=30) (reference group).

Variable	β (95% CI)	R^2
Pupillometry		
Pupil constriction		
Crude	0.16 (-0.27, 0.59)	0.007
Adjusted model[A]	2.86 (-1.18, 6.89)	0.110
Adjusted model[B]	2.98 (-0.83, 6.80)	0.219
Swimming[C]	**-9.39 (-15.40, -3.37)***	
Cross-country skiing[C]	-4.79 (-10.19, 0.60)	
Pupil amplitude		
Crude	-0.35 (-8.30, 7.60)	0.000
Adjusted model[A]	0.57 (-7.12, 8.28)	0.102
Adjusted model[B]	3.26 (-0.65, 7.17)	0.180
Swimming[C]	**-8.63 (-14.83, -2.44)***	
Cross-country skiing[C]	-3.88 (-9.24, 1.51)	
Average pupil constriction velocity (ACV)		
Crude	-3.10 (-7.43, 1.14)	0.024
Adjusted model[A]	-2.98 (-7.15, 1.19)	0.110
Adjusted model[B]	3.65 (-0.27, 7.57)	0.195
Swimming[C]	**-8.24 (-14.38, -2.10)***	
Cross-country skiing[C]	-3.87 (-9.24, 1.51)	
Maximal pupil constriction velocity (MCV)		
Crude	-2.92 (-6.25, 1.14)	0.034
Adjusted model[A]	3.47 (-0.52, 7.46)	0.130
Adjusted model[B]	3.57 (-0.34, 7.48)	0.194
Swimming[C]	**-8.04 (-13.17, -1.91)***	
Cross-country skiing[C]	-3.57 (-0.34, 7.48)	
HRV		
Cardiac vagal index (CVI)		
Crude	**-19.67 (-29.26, -2.09)***	0.057
Adjusted model[A]	**0.55 (0.21, 0.88)***	0.086
Adjusted model[B]	**-13.88 (-26.77, -0.99)***	0.182
Swimming[C]	**-8.32 (-13.03, -3.61)***	
Cross-country skiing[C]	-3.09 (-7.70, 1.51)	

[A]Adjusted for age and sex; [B]Adjusted for age, sex and type of sport; [C]Additional effect from being a swimmer or a cross-country skier in comparison to the reference group.
*p-value <0.05.

The cardiac vagal index (CVI) calculated from the four-second exercise test (4sET) was found to be significantly associated with PD_{20met} (Table 3). The statistical model was adjusted for age, sex and type of sport and explained 18.2% of the variation in PD_{20met} (r^2). None of the parasympathetic pupillometry parameters (pupil constriction, pupil amplitude, ACV or MCV) were associated with PD_{20met}. However, after adjusting for the type of sport (swimming or cross-country skiing), the pupillometry variables were all significantly associated with PD_{20met} in swimmers. The associations between BHR and CVI were shown to be stronger in swimmers than in the reference group (non-athletes) and ceased to be significant in cross-country skiers (Table 3).

Group differences in parasympathetic activity and BHR (Paper I)

Athletes with asthma exhibited increased pupil constriction (CON) (p=0.002) in comparison to healthy athletes (Table 4). Non-athletes also showed increased CON in comparison to healthy athletes (p<0.001). However, healthy athletes had increased initial and minimal pupil diameter values in comparison to both asthmatic athletes (p<0.01) and non-athletes (p<0.01). Other parasympathetic variables obtained from pupillometry and CVI did not differ between asthmatic athletes, healthy athletes and non-athletes. No differences in pupillometry parameters or CVI values were found between subjects who were using inhaled corticosteroids and subjects who were not (data not presented). We found no differences in any of the parasympathetic activity parameters between subjects grouped by PD_{20met} cut-off values of 2, 8, and 16 μmol (data not presented).

Table 4 Parasympathetic parameters, presented as means with 95% confidence intervals, from pupillometry and a four-second exercise test (4sET) in asthmatic athletes, healthy athletes and healthy non-athletes.

Test	Parameters	Asthmatic athletes (n=30)	Healthy athletes (n=27)	Non-athletes (n=30)
Pupillometry	Initial diameter (mm)	6.5 (6.3, 6.7)	**6.9 (6.8, 7.1)*†**	6.4 (6.1, 6.8)
	Min diameter (mm)	4.5 (4.3, 4.7)	**5.0 (4.8, 5.2)*†**	4.4 (4.1, 4.7)
	Amplitude (mm)	2.0 (1.9, 2.1)	1.9 (1.8, 2.0)	2.0 (1.9, 2.1)
	Constriction (%)	30.7 (29.1, 32.2)	**27.7 (26.5, 28.8)*†**	32.1 (30.3, 33.9)
	MCV (mm/s)	5.4 (5.1, 5.6)	5.3 (5.1, 5.4)	5.6 (5.4, 5.9)
	ACV (mm/s)	4.1 (3.9, 4.3)	3.9 (3.8, 4.0)	4.2 (4.0, 4.4)
HRV	CVI#	1.44 (1.39, 1.50)	1.41 (1.35, 1.46)	1.38 (1.32, 1.44)

Min, minimum; MCV, maximal constriction velocity; AVC, average constriction velocity; HRV, heart rate variability; CVI, cardiac vagal index. #n=31 asthmatic athletes, 29 healthy athletes and 29 non-athletes. *Different from non-athletes; †Different from asthmatic athletes (P<0.05; Tukey's HSD post hoc test).

Cross-country skiers showed decreased mean percent pupil constriction (CON) and minimal (min) pupil diameter in comparison to non-athletes but not in comparison to swimmers (Table

5). No other differences in parasympathetic parameters were found between swimmers and cross-country skiers or non-athletes.

Table 5 Parasympathetic activity variables, presented as means with 95% confidence intervals (CI), from pupillometry, and heart rate variability (HRV), measured at the onset of exercise in competitive swimmers, cross-country skiers, elite (cross-country skiers) and healthy non-athletes.

	Parameters	Swimmers (♂17:♀12)	Cross-country skiers (♂18:♀10)	Non-athletes (♂14:♀16)
Pupillometry	Initial diameter (mm)	6.7 (6.5, 6.9)	6.9 (6.6, 7.1)	6.4 (6.1, 6.8)
	Min diameter (mm)	4.7 (4.3, 5.0)	**4.9 (4.6, 5.1)***	4.4 (4.1, 4.7)
	Amplitude (mm)	2.0 (1.9, 2.1)	2.0 (1.9, 2.1)	2.0 (1.9, 2.1)
	Constriction (%)	30.0 (28.1, 31.7)	**29.0 (27.4, 30.6)***	32.1 (30.3, 33.9)
	MCV (mm/s)	5.4 (5.1, 5.6)	5.5 (5.3, 5.7)	5.6 (5.4, 5.9)
	ACV (mm/s)	4.0 (3.8, 4.2)	4.1 (4.0, 4.3)	4.2 (4.0, 4.4)
HRV	CVI	1.42 (1.36, 1.48)	1.42 (1.37, 1.47)	1.38 (1.31, 1.44)

Min, minimum; MCV, maximal constriction velocity; AVC, average constriction velocity; HRV, heart rate variability; CVI, cardiac vagal index. *Different from non-athletes ($p<0.05$).

Bronchial hyperresponsiveness (BHR)

Clinical BHR (PD_{20met} ≤8 μmol) was found in 67% of the asthmatic athletes, 58% of the healthy athletes and 33% of the non-athletes included in study I. The distribution of PD_{20met} differed among groups ($p=0.005$) but not between asthmatic and healthy athletes ($p=0.065$) (Figure 5).

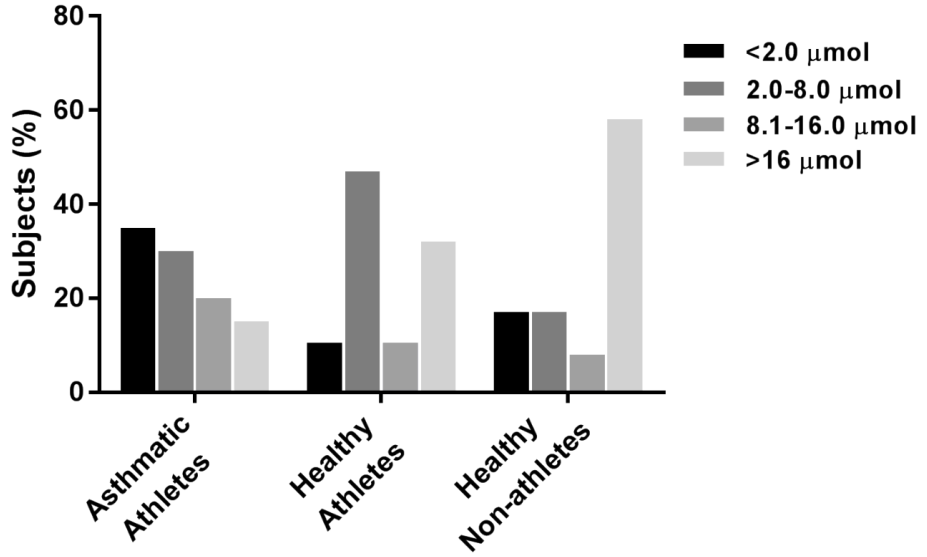


Figure 5 Severity of bronchial hyperresponsiveness (BHR), defined as the methacholine dose (μmol) causing a ≥20% decrease in forced expiratory volume in one second (FEV1) (PD20met), in 20 athletes with asthma, 19 healthy athletes and 24 healthy non-athletes. The distribution of PD_{20met} differed among groups (p=0.005).

The distribution of PD_{20met} (Figure 6) differed significantly between swimmers, cross-country skiers and non-athletes ($p<0.05$). Severe BHR was significantly more frequent in swimmers than in cross-country skiers. Fourteen swimmers (48%) had severe BHR ($PD_{20met} \leq 2$ μmol) in comparison to only one cross-country skier ($p<0.001$). In addition, 72% of the swimmers had clinical BHR ($PD_{20met} \leq 8$ μmol), in comparison to 42% of the cross-country skiers and 39% of the non-athletes ($p=0.015$). The swimmers were younger than the cross-country skiers and elite skiers ($p<0.001$) and trained more hours per week than the cross-country skiers ($p<0.001$). PD_{20met} did not correlate significantly with training hours/week ($\varrho=-0.25$, $p=0.08$). The percentage of subjects with severe BHR ($PD_{20met}<2$ μmol) or clinical BHR ($PD_{20met}<8$ μmol) did not differ between asthmatic and non-asthmatic subjects, nor between subjects who were using inhaled corticosteroids and subjects who were not (data not presented).

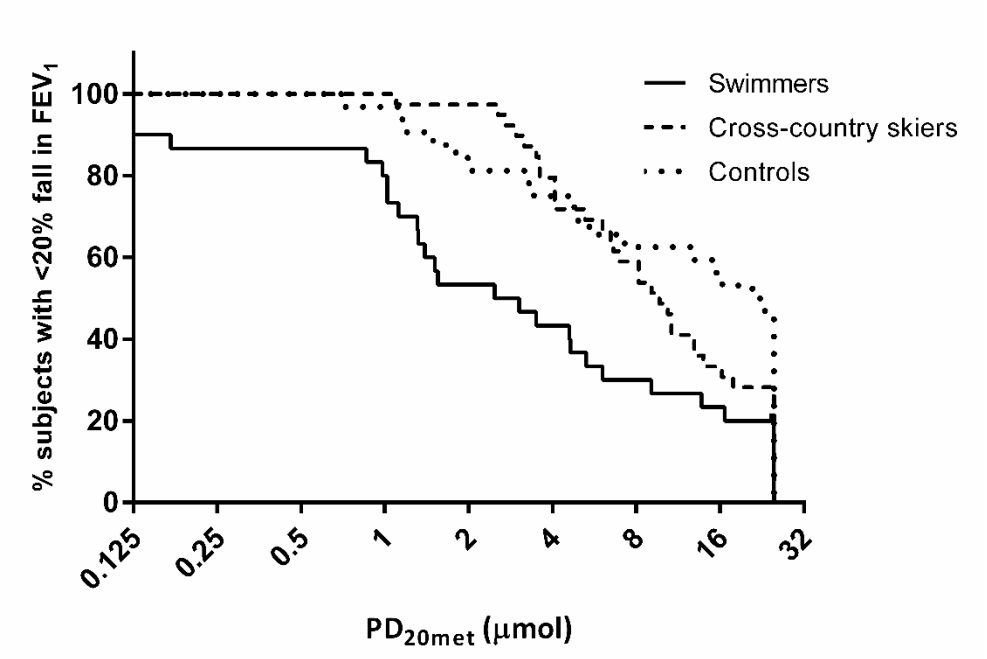


Figure 6 Kaplan-Meier plot showing the distribution of the provocation dose of accumulated inhaled methacholine (PD_{20met}) causing a ≥20% reduction in forced expiratory volume in one second (FEV_1) in cross-country skiers, swimmers and healthy non-athletes. All Kaplan-Meier curves are differed significantly from each other (p=0.005).

Airway inflammation in athletes with and without asthma (Paper II)

A total of 26 swimmers, 27 cross-country skiers and 27 non-athletes from study I completed sputum induction. Fourteen subjects (18%) were unable to produce sputum with a sufficient cell content. After cytospin cell counting, three samples (two control subjects and one healthy cross-country skier) were excluded due to the presence of a large number of squamous epithelial cells (>50% of total cells). Thus, the total sample included in the present study included 63 subjects, with 10 swimmers (♂5/♀5) and 10 cross-country skiers (♂8/♀2) meeting the criteria set for current asthma. The asthmatic athletes and the healthy athletes exercised the same number of hours per week. However, the swimmers (22.2 hours [20.8, 23.6], mean [95%CI]) trained more hours than the cross-country skiers (14.3 hours [12.8, 15.8]) ($p<0.001$). The participants in the control group were older than the participants in both athlete groups ($p<0.001$). No differences in the prevalence of atopy were observed between the groups.

The total sputum cell counts were similar among the three groups, and no significant differences in the percent of non-squamous cells or the total number of each type of inflammatory cell per gram of sputum were observed (Table 7). For all subjects, eosinophils represented ≤2% of the total cell counts. Epithelial cells in induced sputum varied from 1.2 to 2.0 percent of the total cells, with no significant differences between groups. No differences in percent sputum inflammatory or epithelial cell counts were observed between subjects with or without BHR (defined as PD_{20met} <8 μmol, <4 μmol or <2 μmol). Sputum inflammatory or epithelial cell counts did not correlate with PD_{20met}. No significant correlations were observed between training hours per week or years of sport participation and sputum neutrophils or epithelial cells, respectively, among the athletes. Non-atopic subjects (n=39) showed inflammatory cell counts similar to those observed for the 26 atopic subjects (9 asthmatic athletes, 6 healthy athletes and 11 non-athletes).

Table 6 Differential cell counts in induced sputum (presented as proportion (differential %) and absolute numbers) and protein markers from swimmers and cross-country skiers with and without asthma and healthy non-athletes given in medians (25th to 75th percentiles) unless otherwise stated.

	Asthmatic athletes			Healthy athletes			Non-athletes
	All (n=20)	Swimmers (n=10)	Cross-country skiers (n=10)	All (n=19)	Swimmers (n=10)	Cross-country skiers (n=9)	(n=24)
Neutrophil granulocytes							
%*	38 (27-50)	34 (18-50)	42 (23-60)	36 (27-44)	38 (29-48)	31 (14-48)	31 (22-40)
Airway macrophages							
%*	60 (50-72)	65 (49-81)	57 (38-75)	63 (55-72)	61 (51-70)	68 (51-85)	68 (58-77)
Lymphocytes							
%*	1.2 (0.6-1.8)	1.0 (0.4-1.6)	1.4 (0.4-2.5)	0.9 (0.6-1.2)	0.9 (0.5-1.4)	0.8 (0.4-1.2)	1.0 (0.7-1.3)
Eosinophils							
%*	0.2 (0.0-0.4)	0.1 (0.0-0.2)	0.3 (0.0-0.7)	0.1 (0.0-0.2)	0.1 (0.0-0.3)	0.2 (0.0-0.3)	0.1 (0.0-0.3)
Protein markers							
Sputum IL-8 (pg/ml)	378 (167-1123)	462 (169-1737)	356 (161-787)	340 (176-892)	863 (195-1127)	194 (168-446)	217 (130-314)
Sputum IL-1β (pg/ml)	9.6 (6.1-30.8)	10.2 (5.7-41.3)	8.9 (6.5-15.7)	12.6 (9.7-20.0)	13.1 (11.2-21.6)	11.6 (7.0-20.4)	9.0 (5.7-18.2)
Sputum CC16 (ng/ml)	2208 (642-4907)	2701 (635-6588)	2208 (959-3856)	2775 (871-3813)	3292 (1505-3974)	1837 (767-2847)	1332 (489-4043)
Plasma CC16 (ng/ml)	8.1 (6.3-9.6)	6.5 (3.3-8.1)	8.8 (7.8-10.4)	6.2 (5.3-8.3)	5.7 (4.3-9.4)	6.2 (5.4-7.7)	7.5 (6.5-8.8)

*Data presented as means (95% confidence intervals). †Leukocytes. CC16, Club Cell protein 16; IL, interleukin.

Both athlete groups had increased IL-8 in comparison to non-athletes (p=0.02) (Figure 7). However, no significant differences in sputum IL-1β were observed (Table 6). Neither IL-1β nor IL-8 correlated with PD_{20met}. However, sputum neutrophils (%) correlated with both IL-1β (ϱ=0.389, p=0.002) and IL-8 (ϱ=0.481, p<0.001). No differences in either sputum or plasma CC16 were observed between asthmatic and non-asthmatic subjects. Neither sputum nor plasma CC16 correlated with PD_{20met} or sputum inflammatory or epithelial cell counts. However, sputum CC16 correlated inversely with years of sports participation (ϱ=-0.367, p=0.039) among athletes. A weak correlation between sputum and plasma CC16 was observed (ϱ=0.281, p=0.024).

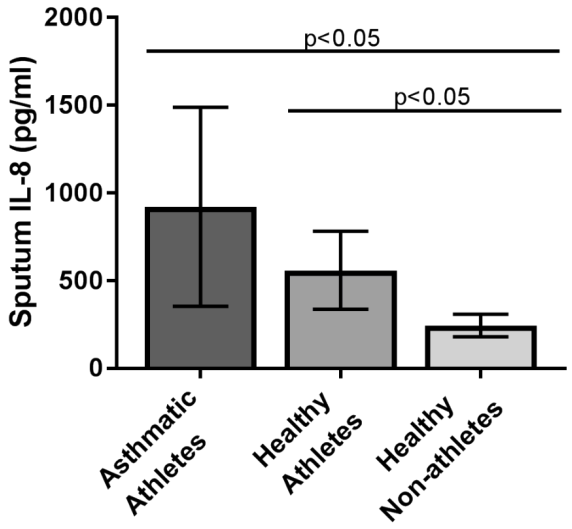


Figure 7 Sputum interleukin (IL)-8 in athletes with asthma (n=20), healthy athletes (n=19) and healthy non-athletes (n=24), presented as the median with the interquartile range. The error bars represent the maximal and minimal values. *Significantly different from non-athletes (p<0.05).

Asthma phenotypes (Paper III)

We identified two distinct asthma phenotypes in elite-level athletes based on the nine asthma variables included in the analysis (Table 2). Class I ("atopic asthma") was characterized by the occurrence (%) of atopy (a positive SPT), increased levels of FE_{NO} (>20 ppb) reflecting eosinophilic airway inflammation, and the presence of allergic rhinitis or other allergic co-morbidities. Class II ("sports asthma") was characterized by the presence of exercise-induced respiratory symptoms and BHR in the absence of allergic features. In our study sample, 104 of 150 athletes (69%) were assigned to class I ("atopic asthma) and 46 athletes (31%) were assigned to class II ("sports asthma"). The percentages of athletes with respiratory symptoms, airway

hyperresponsiveness, a positive reversibility test (reversibility) and current use of asthma medications (therapeutic) were somewhat similar between classes (Figure 8). A higher percentage of athletes with airflow obstruction ($FEV_1/FVC < 0.7$) was observed in athletes with "sports asthma" than in athletes with "atopic asthma" (Figure 8).

The majority of the athletes included were diagnosed with asthma based on the occurrence of bronchial hyperresponsiveness (BHR) after a provocation challenge (n=105 of 150 athletes). Of these subjects, 101 athletes performed a methacholine bronchial challenge. Forty-five athletes were diagnosed with asthma based on positive bronchodilation, and the mean increase in FEV_1 post-inhalation of a bronchodilator was 13% ±9.4 or 450 mL ±292.

An increased risk of "sports asthma" was found among athletes practicing "water sports" (Odds ratio 2.87 [95% CI: 1.82, 4.51]) and "winter sports" (Odds ratio 8.65 [2.67, 28.03]) in comparison to athletes practicing "other sports".

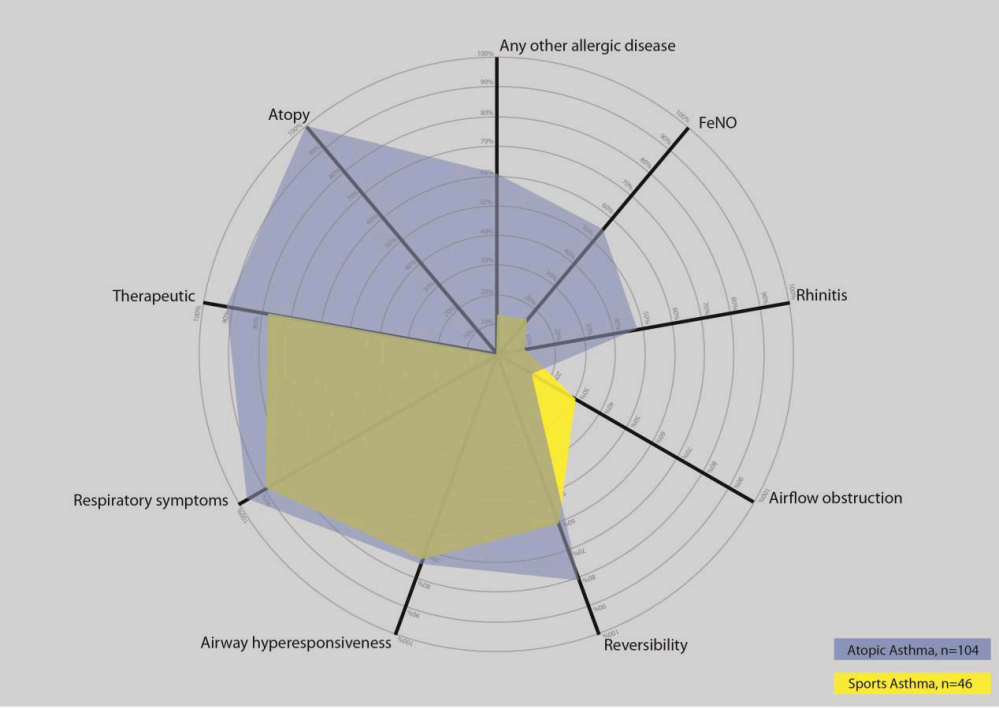


Figure 8 Percentage of athletes presenting each of the variables included in the Latent Class Analysis (LCA). Airflow obstruction was defined as an FEV_1/FVC ratio of <0.70. Reversibility was defined as an increase in FEV_1 of at least 12% and 200 mL. Airway hyperresponsiveness was defined as a positive provocation test using exercise, eucapnic voluntary hyperventilation or the inhalation of 4.5% natrium chloride (NaCl), mannitol or methacholine, according to the International Olympic Committee. Asthma treatment (therapeutic) included current or recent use of inhaled corticosteroids and/or bronchodilators, such as β_2-agonists or ipratropium bromide. Eosinophilic inflammation was defined as fractional exhaled nitric oxide (FeNO) >25 ppb. Reprinted with permission from the Taylor & Francis Group: [J Asthma] Couto et al. 2015;52(9):897-904, copyright 2015.

Discussion

Overall, the present thesis aimed to increase our understanding of asthma mechanisms in athletes and specifically set out to investigate whether increased parasympathetic activity influences BHR in competitive swimmers and cross-country skiers, as well as to characterize airway inflammation in these athletes. In addition, a statistical model was used to pool and identify clusters of clinical features in elite athletes with asthma.

The main results showed that the association of BHR with parasympathetic activity was dependent on the measurement procedure (or target organ), as well as the type of sport (Paper I). A negative association between PD_{20met} and CVI suggests that a high CVI, representing increased parasympathetic activity, is related to more severe BHR, as shown by a lower PD_{20met}. No associations were found between pupillometry variable and PD_{20med}. This finding may suggests that parasympathetic activity measured in the heart and the lungs is more closely related to BHR than parasympathetic activity measured in the pupils and lungs. However, then adjusted for type of sport, associations between pupillometry and PD_{20met} were present in swimmers. A severe PD_{20met} was found more frequently in swimmers than cross-country skiers, which may have influenced these associations. No difference in sputum inflammatory cell counts was found between asthmatic athletes, healthy athletes and non-athletes (Paper II). However, increased sputum IL-8 was present in both asthmatic and healthy athletes in comparison to healthy non-athletes. Airway inflammatory cells or markers did not correlate with PD_{20met}. Lastly, two distinct phenotypes were identified in elite athletes, and an increased risk of "sports asthma," as opposed to "atopic asthma," was found among athletes who competed in water and winter sports (Paper III).

These findings support the hypothesis that increased parasympathetic activity is related to BHR in swimmers and cross-country skiers and also show that the measurement procedure must be taken into consideration. Furthermore, the results are consistent with previous studies that suggested that "sports asthma" in athletes is related to the training environment and/or the type of training and thus is mediated by different mechanisms than asthma in non-athletes. The results obtained in the present thesis may contribute to better understanding of asthma in athletes, better diagnostic tools and the development of targeted treatments (i.e., designed for specific phenotypes). However, several issues have arisen, which must be discussed.

Mechanisms of asthma in athletes

The role of the parasympathetic nervous system in athlete's asthma

It has been hypothesized that the high prevalence of asthma and BHR in endurance-trained athletes may be attributed to an "autonomic dysfunction" (Moreira et al., 2011; Park et al., 2008). Increased parasympathetic activity is shown by HRV indices in endurance-trained subjects and is found to correlate with $\dot{V}O_{2max}$ (Buchheit & Gindre, 2006; Goldsmith et al., 1997). This finding suggests that endurance training and increased aerobic capacity are accompanied by increased parasympathetic activity. Furthermore, increased parasympathetic parameters in pupillometry have been found in long-distance runners (Filipe et al., 2003; Kaltsatou et al., 2011), showing that the increased parasympathetic activity extends beyond the cardiovascular system. The evidence presented in the current thesis supports this theory and the hypothesis that BHR in endurance athletes is related to increased parasympathetic activity. The associations between BHR to methacholine and CVI (and parasympathetic pupillometry parameters in swimmers) suggest that parasympathetic activity plays a role in causing bronchial obstruction and asthma symptoms. However, these associations were less consistent after adjusting for the type of sport. Nevertheless, this finding is supported by the work of Knöpfli and co-workers, who previously demonstrated a relationship between cardiac vagal activity (CVI) and parasympathetic bronchial tone, measured as the protective effect from inhaled ipratropium bromide during exercise, in both runners (1999) and children with EIB (2005).

The present thesis somewhat failed in identifying consistent differences in parasympathetic activity between athletes with and without asthma (Table 4) or between subjects classified by cut-off points of PD_{20met} (BHR severity). Although athletes with asthma showed an increased percentage of pupil constriction in comparison to healthy athletes, the healthy non-athletes had an even higher percentage of pupil constriction. No group differences were found in cardiac vagal activity (CVI) measured during a 4 second exercise test. This finding conflicts with studies that found increased parasympathetic pupillometry variables in athletes in comparison to non-athletes (Filipe et al., 2003; Kaltsatou et al., 2011), although Filipe et al. reported increased pupil constriction only after stratifying for the type of sport and only in long-distance runners (and not in swimmers). Moreover, in the present studyincreased initial and minimal pupil diameter values were found in healthy athletes in comparison to asthmatic athletes and non-athletes (Table 4). The initial and minimal pupil diameter during pupillometry are suggested to reflect the sympathetic-parasympathetic balance (Filipe et al., 2003), and the initial diameter may influence

pupil constriction (Fountas et al., 2006). Therefore, our results must be interpreted with care. Couto et al. (2015) found increased pupil constriction in swimmers with severe BHR (PD_{20met}<2 μmol) in comparison to swimmers with no or mild BHR, and a correlation between pupil constriction and PD_{20met} was also found. This finding conflicts with the results in paper I. Nevertheless, the study of Couto and colleagues is limited by a small sample size, with only two swimmers showing a PD_{20met} <2 μmol. Moreover, the study showed no significant differences between asthmatic and healthy swimmers, which is consistent with the results presented in paper I. This issue is further discussed in the Methodological Considerations section.

Airway inflammation and epithelial damage in athletes

In study I, we sampled induced sputum from athletes (and non-athletes) in a basal state, meaning that the participants had not exercised that day. Our results will therefore reflect not the acute response to exercise but rather the long-term state of the lungs in competitive endurance athletes who exercise >10 hours per week. The results show that airway inflammatory cells are not increased in athletes >12 hours after exercise and are consistent with other studies that showed no to minimal evidence of airway inflammation in swimmers and winter sport athletes (Bougault et al., 2009) and young swimmers (Pedersen et al., 2008). In a similar study, a mild neutrophilic inflammation was present in water-sport athletes, both with and without asthma (Belda et al., 2008). In paper II, we found increased sputum IL-8 in both athlete groups in comparison to the non-athletes, and a correlation with neutrophils in induced sputum was found. IL-8 is a chemokine produced by inflammatory or epithelial cells, and increased sputum IL-8 is found in athletes after long-distance running (Bonsignore et al., 2001; Chimenti et al., 2010), as well as after a submaximal exercise test during a competitive season (Denguezli et al., 2008). Increased IL-8 was found in swimmers with BHR, but not in swimmers without BHR, after a swim ergometer sprint (Kalsen, Hostrup, Bangsbo, & Backer, 2014), which suggests a relationship between IL-8 and BHR. The swimmers included in the present study had a marked increase in BHR in comparison to both cross-country skiers and non-athletes, yet similar sputum inflammatory cells were found. This finding is consistent with a study performed by Martin and colleagues (2012), who found a marked increase in BHR in swimming pool-based athletes in comparison to non-pool based athletes, yet sputum eosinophils or neutrophils were not increased in either group. Based on these data, one may speculate that the frequent occurrence of BHR and respiratory symptoms in endurance athletes may be more associated with increased parasympathetic bronchial tone than persistent airway inflammation. However, the relationship

between BHR and airway inflammation, particularly the role of IL-8 in endurance athletes, need to be investigated further.

Epithelial damage is suggested to be an important feature of athlete's asthma, and there is evidence of airway damage and airway remodelling in bronchial biopsies from cross-country skiers (Sue-Chu, Larsson, Moen, Rennard, & Bjermer, 1999; Karjalainen et al., 2000), exercising mice (Chimenti et al., 2007) and racing Alaskan sled dogs (Davis et al., 2002). It is suggested that higher counts of epithelial cells in induced sputum are a sign of bronchial epithelial damage (Hallstrand et al., 2005). Increased sputum epithelial cells were reported in long-distance runners after a competition (Bonsignore et al., 2001; Chimenti et al., 2010), as well as in swimmers in the basal state (Bougault et al., 2009), irrespective of asthma diagnosis. However, in paper II, we did not find increased sputum bronchial epithelial cells in our study sample. Furthermore, we found no increased levels of sputum or plasma CC16, which has been used as a marker of epithelial damage in athletes (Bernard, Carbonnelle, Nickmilder, & de Burbure, 2005). This finding may be explained by the fact that our participants had not exercised on the day of testing.

Asthma phenotypes in athletes

It is suggested that there are two main asthma phenotypes in athletes (Haahtela et al., 2008). Asthma may be present from early childhood, characterized by atopy and signs of bronchial eosinophilic inflammation, with increased FE_{NO} and with symptoms consisting of periods of exacerbation, with chest tightness and wheezing. In the second phenotype however, symptoms like cough and phlegm provoked by exercise and viral infections are described, along with evidence of increased neutrophilic inflammation and bronchial epithelial damage. Athletes of the latter phenotype develop asthma through participation in sports, and this phenotype is suggested to be associated with "autonomic dysautonomy" induced by systematic high-intensity endurance exercise (Moreira et al., 2011). The phenotypes that were identified in study II correspond to the phenotypes suggested by Haahtela and colleagues (2008). The "atopic asthma" phenotype was characterized by the occurrence of atopy, increased FE_{NO} and other allergic co-morbidities, while the "sports asthma" phenotype was defined by the presence of exercise-induced respiratory symptoms and BHR in the absence of allergic features. Interestingly, the "sports asthma" phenotype was characterized by a reduced FEV_1/FVC ratio, possibly induced by increased parasympathetic bronchial tone (Figure 8). The type of sport or the specific type of exercise and environmental conditions were associated with an increased risk of this phenotype, as winter or water sport athletes had an increased risk of "sports asthma" in comparison to other athletes. "Sports asthma"has been compared to the late-onset phenotype identified among non-athlete

asthmatics (Peters, 2014). In many cases, this late-onset phenotype appears to be less responsive to standard therapy and more related to environmental risk factors (Peters, 2014). Large training volumes in unfavourable environments can either worsen existing asthma or lead to the development of novel disease in a previously healthy but susceptible athlete. It has been suggested that competitive athletes with atopic asthma may shift to the "sports asthma" phenotype after years of sports participation (Carlsen, Hem, & Stensrud, 2011). In fact, it is debated whether "sports asthma" should be classified as an occupational disease (Price, Ansley, Menzies-Gow, Cullinan, & Hull, 2013).

Differences between sports types

Differences in asthma prevalence and the risk of asthma have been reported in athletes within different types of sports (i.e., endurance sports, strength or power sports, team sports or technical sports) (Helenius et al., 1997; Fitch, 2012). However, it is not clear if asthma development occurs through different mechanisms across different types of sports. The results from paper I indicate that there are differences in parasympathetic activity and BHR between swimmers and cross-country skiers. When adjusted for the type of sport, differences in the associations of parasympathetic parameters with PD_{20met} became apparent, with the associations being significant in swimmers and not in cross-country skiers (Table 3). In addition, the associations between parasympathetic activity and BHR seem to be stronger in swimmers than in cross-country skiers. This finding may be explained by the type of training, the training volume or the training environment. However, a more severe BHR seems to be characteristic in swimmers (Figure 6), which may influence the associations found and may suggest that there is an association between parasympathetic activity and BHR in subjects with severe BHR. On the other hand, no differences in parasympathetic activity were observed in subjects stratified by PD_{20met} cut-off points (2, 4 or 8 μmol). Couto and co-workers (2015) reported that swimmers with severe BHR (<2 μmol) had an increased percentage of pupil constriction by pupillometry and observed a correlation between percent pupil constriction and PD_{20met} However, this study was limited by a small sample size, and no such correlations were found in our present study (paper I). No differences were found in sputum cell counts or protein markers in sputum or plasma between asthmatic and non-asthmatic swimmers and cross-country skiers (Table 6). FE_{NO} was increased in cross-country skiers as compared to swimmers. However, two cross-country skiers had a FE_{NO} >50 ppb, one of whom one had allergy, which influence the mean in this group. There were no differences in the occurrence of atopy between sport types. The use of inhaled corticosteroids may influence both eosinophilic airway inflammation and BHR (Fujimoto

et al., 2006) and was more frequent among cross-country skiers than swimmers. However, no differences in parasympathetic activity or sputum inflammatory cells were found between athletes who were using inhaled corticosteroids and athletes who were not.

The athletes included in the study I represent two sports performed in different types of environments. The cross-country skiers inhale dry air at subfreezing temperatures, and the swimmers exercise in warm and humid environments. Both sports types involve whole-body exercise, but different training intensity and duration or breathing patterns may affect the impact of endurance exercise upon the airways. Differences in training volume are observed between swimmers and cross-country skiers or other cold-air athletes (Bougault et al., 2010), which may affect the development of asthma, as the time between training bouts will decrease, allowing less time for restitution (Carlsen et al., 2011). The different environmental exposures between the two types of sports should also be taken into consideration. Exposure to increased levels of chloride and chloride compounds may be more harmful to the airways than cold air exposure. Furthermore, years of sports participation will also influence the exposure time, with more accumulated training hours (Stensrud et al., 2007), and as swimmers increase their training volume earlier than athletes within other types of sports. These findings need further clarification regarding the mechanisms of asthma development, particularly in swimmers, for whom the occurrence of asthma and allergy have been shown to be very high.

Is it asthma?

The GINA definition of asthma applies to the general population and does not comply as well with observations in athletes. For instance, there is a lack of associations between self-reported respiratory symptoms and objective findings, and conflicting reports are published regarding the presence or persistence of airway inflammation in athletes (Bougault et al., 2009; Pedersen et al., 2008; Sastre et al., 2013; Martin et al., 2012). In addition, FE_{NO}, which is a known objective marker of airway eosinophilic inflammation, is not reported to be a useful tool in athlete's asthma, as few athletes show increased FE_{NO} levels (>20 ppb), which suggests the absence of airway eosinophilic inflammation (Voutilainen et al., 2013). Furthermore, endurance athletes, particularly swimmers, have increased lung function in comparison to non-athletes (Armour, Donnelly, & Bye, 1993), making it challenging to uncover potential airflow limitations or reduced lung function. Similar findings were observed in the studies included in the present thesis. The results from Paper III suggest that "sports asthma" differs from "atopic asthma" in terms of clinical characteristics, whereas athletes with "atopic asthma" show clinical findings previously reported in non-athletes with asthma, such as increased FE_{NO}. One might speculate that the

athletes with "sports asthma" would not have BHR or report respiratory symptoms without their sports participation and training regimes. As the elite athlete can obtain workloads accompanied by higher ventilation rates than non-athletes, it can be difficult to determine whether to define their responses to these extreme strenuous performances as pathological. Furthermore, BHR is also frequently observed in healthy athletes (Langdeau & Boulet, 2001). Knöpfli and co-workers (2005) suggested that the variation in the presence of asthma and BHR between athletes competing in different sports or individuals within the same sport may be attributed to differences in parasympathetic activity and related to levels of physical fitness ($\dot{V}O_{2max}$). The results from the present thesis suggest that asthma-like symptoms and BHR (measured by methacholine) in swimmers and cross-country skiers are partially caused by increased parasympathetic tone.

In study I, we found no significant difference in the prevalence of BHR between athletes with asthma and healthy athletes. Furthermore, no differences in sputum inflammatory cells or other markers of airway inflammation were found. However, the high prevalence of BHR and respiratory symptoms in athletes without physician-diagnosed asthma suggests that abnormal airway responses are common in athletes and may imply that high-level endurance exercise is associated with BHR (Langdeau & Boulet, 2001). The lack of airway inflammation in the asthmatic athletes may be due to anti-asthmatic treatment. Of the athletes included in paper II, 43% used inhaled corticosteroids. Five swimmers and two cross-country skiers were unaware of having asthma when entering the study (study I), but were diagnosed with asthma after clinical examination and performance of objective tests, exemplifying the importance of objective tests used to diagnose asthma in athletes.

Methodological considerations

Measurement procedures for parasympathetic activity

Multiple methods and protocols have been used to measure parasympathetic activity in human subjects. The tests used in the present studies were chosen based on previous reports on athletes (Filipe et al., 2003; Araújo et al., 1989; Knöpfli & Bar-Or, 1999).

Target organ

The present thesis aimed to examine if the target organ of measurement for parasympathetic activity influenced the relationship between BHR and parasympathetic activity in athletes. The results showed that the association differs between the HRV and pupillometry tests. This lack of agreement between these procedures may suggest that measurements of parasympathetic activity

should be targeted to the specific organ of interest. A weak but significant association was found between CVI, but not pupillometry variables and PD_{20met}, before adjusting for type of sport. This association may occur because both the heart and the lungs are served by the vagus nerve, and bronchoconstriction is mediated, like bradycardia, by afferent nerves in the n. vagus, allowing a link between the regulation of the heart and the bronchi (Levy, 1997; de Jongste et al., 1991; Grossman & Taylor, 2007). However, Horvath and colleagues (1995) found no agreement between HRV and resting specific airway resistance (sR_{AW}), which they suggested to represent the vagal activity of the bronchi. These findings may reflect the fact that although the parasympathetic regulation of the heart and the bronchi is mediated through the n. vagus, neurogenic differences exist between these organs (for instance, regarding neural pathways, receptor sensitivity or the regulation of sympathetic-vagal balance). In addition, the parasympathetic bronchial tone, as shown sRaw in the study by Horvath and colleagues (1995) or the cholinergic sensitivity to inhaled methacholine in the present study, may reflect different aspects of the autonomic regulation of the bronchi.

Validity of the methods

Although many studies suggest that asthmatic subjects have increased cardiac parasympathetic activity and exaggerated bronchomotor sensitivity to muscarinic agonists, the evidence is conflicting. The reason for this variability may be the lack of specific measurement procedures for parasympathetic activity in the bronchi. The sensitivity and the specificity of these tests with respect to asthma and BHR have not been determined. Our results suggest that measurements of parasympathetic activity in different target organs do not compare. Thus, pupillometry and HRV may be poor tools for assessing parasympathetic bronchial tone in athletes. Still, in paper I, there was a negative association between CVI and PD_{20met} in a sample consisting of asthmatic and healthy cross-country skiers and swimmers and non-athletes. This finding suggests that HRV is a more appropriate tool for evaluating asthmatic athletes than pupillometry. These findings are supported by two studies from Knöpli and colleagues who found a high correlation between CVI and reversibility to inhaled ipratropium bromide (Knöpfli et al., 2005; Knöpfli & Bar-Or, 1999).

In study I, the 4sET protocol was chosen primarily because of the findings reported by Knöpfli and colleagues (Knöpfli et al., 2005; Knöpfli & Bar-Or, 1999). This test has also been found to be comparable to other methods for cardiac vagal assessment, including the time and frequency domains of HRV, as well as RSA, in healthy subjects (Paiva et al., 2011). However, in contrast to other studies on HRV (Goldsmith et al., 1997; Buchheit & Gindre, 2006), the results from the present thesis (paper I) found no differences in cardiac vagal activity between endurance athletes

and non-athletes. These results are consistent with those from a study by Araújo and colleagues (2015), in which the 4sET was performed on 90 athletes and 58 non-athletes. Both studies found a tendency toward increased CVI in athletes, yet no significant differences were found. The results presented in this thesis show large inter-individual variations in CVI (Tables 4 & 5), which may explain the lack of statistically significant group differences. The heart rate is under constant influence from various factors, including hormones (adrenaline, thyroid hormones), breathing patterns, stature, and temperature, suggesting that the heart may be confounded by more factors than the pupil with respect to assessments of autonomic function and that the pupil is a more appropriate target organ. Filipe et al. (2003) found no differences in pupillometry parameters between athletes and non-athletes. However, when stratified by type of sport, endurance-trained runners showed increased pupil amplitude and percent pupil constriction in comparison to soccer players, swimmers and gymnasts, as well as in comparison to sedentary control subjects. Another potential explanation for the lack of significant results in the present thesis is that the non-athletes included in study I exercised regularly, and many reported that they had previously participated in competitive sports. Unfortunately, we did not objectively measure aerobic capacity ($\dot{V}O_{2max}$) or performance; therefore, we can only assume that the athletes were more fit than the non-athletes. Kaltsatou and colleagues (2011) compared pupillometry parameters between endurance- and power-trained athletes and sedentary controls and found increased parasympathetic activity in the endurance trained athletes during rest, exercise and recovery. The parasympathetic branch of the ANS is dominant at rest, while during intensive exercise, the sympathetic system is dominant (for instance, to increase heart rate). In the present study, parameters of parasympathetic activity were assessed at rest, with the exception of the CVI, which was assessed at the onset of a short cycling bout (Araújo et al., 1992). Our results do not assess the autonomic regulation of the bronchi during exercise; therefore, the results presented in the present do not thesis reveal whether an autonomic dysfunction occurs during exercise, leading to EIB or respiratory symptoms, in these athletes. Thus, differences in autonomic regulation during exercise may differ from our observations.

Can we determine parasympathetic activity in the bronchi?

The different associations between BHR and parasympathetic activity measured in different target organs reported in the present thesis suggest that specific measurements of bronchial parasympathetic tone may be required to assess asthma mechanisms. Measurements of parasympathetic activity in the lungs are not often reported in the literature. The airway tone is under parasympathetic influence through the innervation of airway smooth muscle, which can

induce bronchoconstriction when activated or bronchodilatation when inhibited (Mazzone & Canning, 2002; de Jongste et al., 1991). Anticholinergic agents are administered to block the neurotransmitter acetylcholine in the central and peripheral nervous system (Pichon, Roulaud, Denjean, & de Bisschop, 2005). Therefore, the bronchodilating effect of an inhaled anticholinergic drug can be interpreted as a measure of the parasympathetic bronchial tone, as ipratropium bromide inhibits parasympathetic nerve impulses through competitive inhibition of the muscarinic acetylcholine receptors (Sterk & Bel, 1989). In a study performed by Horvath et al. (1995), measures of specific airway resistance (sRaw) were described to reflect the parasympathetic bronchial tone. In this study, no correlation was found between sRaw and HRV or heart rate periods (inter-beat intervals). An appropriate assessment of parasympathetic bronchial function might foster the identification of BHR mechanisms in endurance athletes, monitor the development of this condition and guide treatment. Measurements of bronchial tone or airway resistance during exercise may provide additional information regarding the autonomic balance between the parasympathetic and sympathetic branches during exercise at different intensities, for instance, by tidal volume flow-volume curves (intrabreath) or impulse oscillometry (Verges et al., 2005; Suman, Beck, Babcock, Pegelow, & Reddan, 1999; Price, Ansley, Bikov, & Hull, 2016).

Measurement procedures for BHR

In study I, BHR was assessed using methacholine bronchial challenge, which is a direct test in which methacholine acts directly on receptors in the bronchial smooth muscles to cause contraction (Anderson & Brannan, 2011). Verges et al. (2005) found poor agreement between methacholine and exercise when used as provocation agents for BHR assessments (Verges et al., 2005). Similarly, Holzer and colleagues (2002) compared methacholine with the EVH test in elite summer sport athletes and found poor agreement between the tests; only 9/42 subjects had a positive methacholine test, and 25 subjects had a positive EVH challenge result. The lack of agreement between direct and indirect provocations tests suggests different mechanisms of direct and indirect BHR and that these tests cannot be used interchangeably. Furthermore, it has been stated that the methacholine bronchial challenge, a direct test, is more related to airway remodelling, in contrast to indirect tests, such as exercise tests, the mannitol test and the EVH test, which have been regarded as more related to airway inflammation (Riiser, Hovland, Carlsen, Mowinckel, & Lodrup Carlsen, 2012; Porsbjerg, Brannan, Anderson & Backer, 2008). This finding may indicate that direct and indirect bronchial challenges can supplement each other and reveal different mechanisms and phenotypes of asthma or airway dysfunction in athletes (Price et al., 2016). Therefore, it should be tested if the associations between parasympathetic activity and

airway inflammation or BHR that were reported in the results of the present study exist when BHR is measured with indirect methods, such as EVH, mannitol or an exercise test.

The methacholine bronchial challenge has been shown to be more sensitive in identifying active asthma than indirect tests, such as a standardised exercise test (Leuppi, Kuhn, Comminot, & Reinhart, 1998) and a sport-specific exercise field test (Stensrud et al., 2007), but was shown to be comparable to an EVH test (Stadelmann et al., 2011). The increased sensitivity may be due to increased parasympathetic activity, as previously discussed. The sensitivity and the specificity of bronchial provocation tests are dependent on the criteria used to define a positive test (Hewitt, 2008). Stadelmann and colleagues (2011) found good agreement between the methacholine bronchial challenge and the EVH test when the cut-off dose of methacholine was 4 μmol. However, the cut-off value used by the IOC and WADA until 2010 was 2 μmol in athletes not treated with inhaled steriods (WADA, 2009; Cockcroft, 2010; Carlsen et al., 2008). The cut-off value for methacholine was not mentioned in the study performed by (Holzer, Anderson, & Douglass (2002). In study 1, we used a 8 μmol (1.6 mg) cut-off for BHR (Papers I and II). This is a somewhat higher cut-off than commonly used as recommendation for treatment of asthma in athletes (Carlsen et al., 2008), but is more commonly used in non-athlete asthmatics (Cockcroft, 2010). However, our results did not change when the data was re-analyzed using stricter cut-offs (2 or 4 μmol).

Strengths and limitations

Study design and measurement procedures

The present thesis benefits from the fact that objective tests were provided to accompany self-reported data on respiratory symptoms and asthma diagnosis recorded by questionnaires in all studies. The clinical measurement procedures used were perfromed according to the official guidelines from ERS and ATS (Miller et al., 2005; European Respiratory Society, 1997; Crapo et al., 2000; American Thoracic Society & European Respiratory Society, 2005; Parsons et al., 2013). In study II, we reviewed medical files of Norwegian and Portuguese elite athletes, but it was not possible to identify the type of provocation tests performed by athletes in the two countries. However, all procedures were performed according to IOC criteria. The use of different methods to assess BHR, including both direct and indirect challenges, must be acknowledged as a limitation of paper III because the sensitivity and specificity of these tests may differ. This limitation also applies to the use of only a direct test (methacholine bronchial challenge) in study I, as the method used for BHR assessment might influence the relationship between parasympathetic activity and BHR. To include a indirect test for BHR may have provided

additional information. In addition, we assessed airway inflammation based on induced sputum, which is a recognized sampling method for both monitoring and assessing chronic lung diseases in research and clinical practice (Nicholas & Djukanovic, 2009). A strength of the methods included in the present thesis was that parasympathetic activity was measured in two target organs, allowing for the assessment of different neurogenic pathways of the parasympathetic nervous system. Furthermore, the tests were performed on two different days to limit the risk of error. A limitation of the present thesis is that it only includes cross-sectional studies, which does not permit the identification of causality.

In study I the subjects were grouped based on sport types or asthma diagnosis. Training volume and environmental exposure were not objectively measured, and this may have influenced the results. Moreover, the criteria set for asthma diagnosis may also influence the results. In a study performed by Larsson et al. (1993), which aimed to assess the prevalence of asthma in Swedish cross-country skiers, asthma was defined as current BHR in combination with the presence of two or more (self-reported) symptoms, including cough, abnormal shortness of breath, chest tightness and wheezing. Those authors found that 33% of the skiers fulfilled these asthma criteria. However, ten skiers had asthma diagnosed by a doctor but no BHR. Those participants were thus not asthmatic according to the study criteria, yet they were taking anti-asthmatic agents and had symptoms (Larsson et al., 1993). In the present study, current asthma was defined as a combination of objectively measured BHR, the use of anti-asthmatic drugs and a previous doctor's diagnosis of asthma. However, several healthy athletes and non-athletes had severe BHR ($PD_{20met} \leq 2$ μmol), and several asthmatic athletes (often using ICS) showed no sign of current BHR. Respiratory symptoms during or after exercise are also frequently reported in healthy athletes, as well as in non-athletes, and there might be a risk of a falsely high prevalence of self-reported asthma in this group without performing objective tests. In addition to the fact that BHR may have a low specificity for asthma in athletes (Holzer & Douglass, 2006), the prevalence of asthma might be both over- and under-estimated. In the study performed by Larsson et al. (1993), 80% of skiers reported asthma-like symptoms or BHR or both.

Seasonal variations (i.e., changes in temperature and the release of pollen) are shown to influence BHR and airway inflammation in cross-country skiers (Heir & Larsen, 1995). Furthermore, training volume and intensity will change throughout the year, according to the different phases of the competitive season. These variations may influence BHR (Heir & Larsen, 1995) and possibly $\dot{V}O_{2max}$ and parasympathetic activity. The competitive season of cross-country skiers is from November to March. Swimmers have competitions throughout the entire year, without

lengthy off-season periods. The data collection in study I was carried out throughout a full year; therefore, seasonal variations may have influenced our results, particularly in cross-country skiers. Although we cannot exclude that recent training activity may have influenced BHR, airway inflammation and parasympathetic activity, it is not known if the association between these variables differs over time.

In study II, the asthma phenotypes were established based on an exploratory statistical model to pool and characterize latent classes. However, the statistical analysis was based on nine chosen variables, which were believed to be of clinical importance based on experience and the current literature, as well as the availability of the databases. Other variables may have altered these results, and we could have failed to include other variables of clinical importance. Although the results obtained in this study (paper III) were consistent with a previously published review paper introducing the hypothesis that asthma phenotypes exist in athletes (Haahtela et al. 2008), these phenotypes should be confirmed by replicating the results in other studies, preferably with a prospective design.

Subjects and generalizability

The athletes included in the present studies are all elite or top-national level athletes and are therefore prone to the negative consequences of athlete exercise regimes. In addition, these swimmers and cross-country skiers are exposed to potentially noxious stimuli through the sport-specific environmental conditions of indoor chlorinated swimming pools and cold, dry air, respectively. These factors may limit the generalization of the results to athletes within other sport types. For instance, there is a link between exposure to cold air and increased vagal activity (Araújo, Wilk, Meyer, & Bar-Or, 1994; Deal, McFadden, Ingram, & Jaeger, 1978), as well as EIB (Stensrud et al., 2007), which may be important in cross-country skiers and may not apply to swimmers or other athletes. In addition, swimmers have an extremely high training volume and usually train more than other types of athletes. We found in study I that the swimmers exercised more hours per week than the cross-country skiers, despite being younger. This high training volume affects the athletes' environmental exposure and also reduces the time for restitution (for instance, in the airway epithelium).

The potential influence of the use of anti-asthmatic medications upon the results reported in the present thesis cannot be excluded. The use of ICS may influence airway inflammation, as well as BHR (PD_{20met}), and will therefore confound the associations with parasympathetic activity assessed in paper I. Fifteen percent of the swimmers and 29% of the cross-country skiers

included in this study reported that they used ICS. This factor may also explain why no clear increases in parasympathetic activity or sputum inflammatory cell counts were found in asthmatic athletes in comparison to healthy athletes and non-athletes (papers I & II).

In this field of research, athletes are often compared to other groups, such as sedate or asthmatic subjects or reference materials defining what is "normal." However, it may not always be appropriate to compare athletes with non-athletes. In fact, specific reference values for athletes may be required, such as for lung function or parasympathetic activity. As the non-athletes in study I exercised regularly and had previously been engaged in competitive sports, they might be more comparable to athletes than to subjects who have never participated in competitive sports.

Clinical applications

An increase in the use of anti-asthmatic medications, particularly β_2-agonists, among athletes has been reported (Fitch, 2006). However, significant variability in the airway response to β_2-agonists is observed in athletes, especially among athletes who develop asthma symptoms and BHR during their athletic careers (Carlsen, 2009). The findings presented in the present thesis suggest that measurements of parasympathetic bronchial tone may indicate the susceptibility of the athlete to BHR and the potential benefits of anticholinergic treatment. Although a negative association of CVI with BHR was shown in paper I, it is easier and probably more reliable to assess the parasympathetic activity of the bronchi by measuring the reversibility of the response to anticholinergic drugs directly through lung function measurements before and after inhalation, rather than by assessing the parasympathetic activity of the heart via HRV, even though a link between the vagal activity of the heart and the bronchi was previously shown in patients with BHR (Pichon, de Bisschop, Diaz & Denjean, 2005), children with EIB (Knöpfli et al., 2005) and athletes (Knöpfli & Bar-Or, 1999).

Appropriate asthma treatment is a prerequisite for competitive athletes, as well as for patients. Becker and co-workers (Becker, Rogers, Rossini, Mirchandani, & D'Alonzo, 2004) investigated the rate of deaths caused by asthma in relation to sports and assessed how exercise can trigger a fatal asthmatic attack. This study emphasizes the need for optimal medical treatment for asthmatic athletes, with a focus on controlling anti-inflammatory therapy with specifically trained team physicians, healthcare personnel and coaches and trainers in order to avoid sudden fatal asthma in athletes. The therapeutic effect of ipratropium bromide appears to vary greatly among individuals (Boner, Vallone, & De, 1989; Boulet, Turcotte, & Tennina, 1989), and the evidence for the involvement of the cholinergic system in EIB based on the use of inhaled ipratropium

bromide has varied (Borut et al., 1977; Poppius, Sovijarvi, & Tammilehto, 1986; Boulet et al., 1989; Boaventura, Araujo, Martinez, & Vianna, 2010). This variability may be related to the causes of asthma in individual patients. A general property of endurance athletes may thus be increased sensitivity to the action of anticholinergic medication (i.e., inhaled ipratropium bromide), although this hypothesis was not investigated in the present thesis.

Conclusions

Overall, the results presented in this thesis suggest that BHR, increased parasympathetic activity and the type of sport practiced (training environment or type of training) are involved in the mechanisms of "sports asthma." Different asthma phenotypes exist among athletes, and the risk of asthma is related to the type of sport practiced.

More specifically the conclusions of this thesis are as follows:

1. BHR is associated with cardiac vagal activity in swimmers, cross-country skiers and healthy non-athletes and with parasympathetic variables of pupillometry in swimmers only. The association between BHR and parasympathetic activity in athletes depends on the measurement procedure or target organ, the type of sport practiced and possibly BHR severity.

2. Parasympathetic activity, as measured by HRV and pupillometry, is not increased in asthmatic athletes in comparison to healthy athletes and non-athletes. Group differences in parasympathetic activity, as measured by pupillometry only, were found. However, the clinical value of these differences is not clear.

3. A high prevalence of BHR was found in athletes in comparison to non-athletes, independent of asthma diagnosis. Sputum inflammatory cells were not increased in athletes; however, interleukin-8 in sputum was increased and may be a marker of airway inflammation in athletes. No associations between BHR to methacholine and airway inflammation, as assessed by induced sputum, were found.

4. Two asthma phenotypes were identified in elite athletes: "atopic asthma" and "sports asthma." Athletes who competed in water and winter sports had an increased risk of "sports asthma" in comparison to athletes who competed in other sports.

Future perspectives

The main aim of the present thesis was to increase our understanding of the pathogenic mechanisms of asthma and BHR in endurance athletes, particularly swimmers and cross-country skiers. More knowledge about these mechanisms can contribute to improved (or specific) diagnostic and treatment methods for asthma in athletes, better monitoring of at-risk athletes and the possibility of preventing healthy athletes from developing asthma. However, there is still a need for studies that confirm the causality of the associations presented in this thesis. Whilst the current evidence supports the hypothesis that parasympathetic activity increases in endurance-trained subjects and the hypothesis that this change occurs as a consequence of systematic endurance exercise, it remains unclear whether this effect is causally related to the development of BHR and asthma. Thus, prospective and well-designed studies are needed to establish whether parasympathetic over-activity or dysfunction precedes the onset of symptoms and signs associated with BHR and asthma.

More studies are also required to adjust for individual disposition, environmental factors (exposure), type of sport, intensity of training and other potential confounders. Future works must establish and validate appropriate measurement procedures for parasympathetic bronchial activity both at rest and during exercise. Such studies may subsequently influence the design of clinical trials that use therapeutic interventions with anticholinergic receptor stimulation to establish whether targeting parasympathetic activity leads to improved outcomes for athletes with BHR. Such studies should aim to provide both long- and short-term outcome data on different component features of the state, to identify the parameters that predict responses, and to test and establish new approaches before they can be considered in clinical practice. Until the results from such trials become available, clinicians treating athletes with asthma should be aware that there is a cluster of risk factors associated with asthma and BHR, such as environmental exposure and type of training. Approaches that target these disturbances, such as reducing unfavourable exposure by changing the training environment or adjusting the training load, should be a part of disease management.

Finally, few follow-up studies of athletes with asthma exist, and the persistence or reversibility of "sports asthma" on a long term basis in this population is not known. One study reported evidence that BHR, airway inflammation and asthma are reversible after the end of active sports careers in swimmers. Future follow-up studies should evaluate athletes who compete in different types of sports.

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Paper I

CLINICAL SCIENCES

Parasympathetic Activity and Bronchial Hyperresponsiveness in Athletes

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ABSTRACT

STANG, J., T. STENSRUD, P. MOWINCKEL, and K.-H. CARLSEN. Parasympathetic Activity and Bronchial Hyperresponsiveness in Athletes. *Med. Sci. Sports Exerc.*, Vol. 48, No. 11, pp. 2100–2107, 2016. **Purpose**: A high prevalence of asthma and bronchial hyperresponsiveness (BHR) is reported in swimmers and cross-country skiers. It has been suggested that increased parasympathetic nervous activity is involved in asthma development in endurance athletes. We aimed to assess the associations of BHR to parasympathetic activity in healthy and asthmatic swimmers and cross-country skiers and healthy nonathletes. **Methods**: Parasympathetic activity was measured by pupillometry and heart rate variability at the onset of exercise with the cardiac vagal index calculated in 28 cross-country skiers (♂18/♀10), 29 swimmers (♂17/♀12), and 30 healthy nonathlete controls (♂14/♀16) on two different days. All subjects performed a methacholine bronchial challenge with the provocation dose causing 20% decrease in the forced expiratory volume in 1 s calculated (PD_{20met}). Data were analyzed by robust regression analysis and presented as β coefficients with 95% confidence intervals (CI). **Results**: PD_{20met} was negatively associated with cardiac vagal index (−13.9, 95% CI = −26.8 to −1.0) in all subjects. When adjusted to the type of sport, this association was stronger in swimmers (−8.3, 95% CI = −13.0 to −3.6) as compared with controls and nonsignificant in cross-country skiers. Percent pupil constriction was significantly associated with PD_{20met} in swimmers (−9.4, 95% CI = −15.4 to −3.4) only after adjusting for the type of sport. Fourteen swimmers (48%) and 16 cross-country skiers (57%) had doctor-diagnosed asthma in combination with current BHR and/or current use of asthma drugs. Seventy-two percent swimmers, 44% cross-country skiers, and 39% controls had a $PD_{20met} \leq 8$ μmol ($P = 0.015$). Fourteen swimmers had a $PD_{20met} \leq 2$ μmol as compared with one cross-country skier ($P < 0.001$). **Conclusion**: Parasympathetic activity measured in the heart is more closely related to BHR as compared with parasympathetic activity measured in the pupils. The type of sport influences BHR severity and its relationship to parasympathetic activity. **Key Words:** ASTHMA, ASTHMA MECHANISMS, AUTONOMIC NERVOUS SYSTEM, CROSS-COUNTRY SKIERS, EXERCISE, SWIMMERS

Bronchial hyperresponsiveness (BHR) and asthma are frequently reported in endurance athletes, in particular swimmers and cross-country skiers (9,15,24,33), but the mechanisms of asthma in athletes have not been fully described.

Parasympathetic cardiac activity, measured by heart rate variability (HRV), is shown to be increased in endurance athletes and to correlate with maximal oxygen uptake (6,14,16,37). Furthermore, Filipe et al. (14) found increased pupil constriction measured by pupillometry, reflecting increased parasympathetic activity, in endurance athletes. The autonomic nervous system mediates the contraction and relaxation of bronchial smooth muscle with parasympathetic cholinergic nerves stimulating bronchoconstriction, whereas sympathetic nerves bronchodilate (8). As BHR denotes an increased bronchoconstrictor response to different stimuli, such as cold air, exercise, or pharmacological substances, increased parasympathetic activity could also theoretically predispose an athlete to increased bronchomotor tone and further susceptibility to bronchospasm (27). Indeed, Pichon et al. (30) demonstrated that subjects with BHR had increased parasympathetic indices of HRV from before to after a methacholine bronchial challenge. Methacholine is a synthetic choline ester that acts as a nonselective muscarinic receptor agonist in the parasympathetic nervous system that is frequently used to assess BHR, which is shown to be a sensitive test in athletes (33,36). An inverse correlation between the bronchodilating effect to inhaled ipratropium bromide, but not inhaled salbutamol, a general bronchodilating agent, and BHR to methacholine was found in elite cross-country skiers (34).

Although increasing evidence suggests that BHR to methacholine is related to increased parasympathetic bronchial activity in athletes, this relationship is not fully established. Langdeau et al. (22) found a weak, although significant, correlation between HRV and PD_{20} methacholine and concluded that parasympathetic activity did not explain the differences in BHR prevalence found between swimmers and cold-air athletes. In this study, parasympathetic activity was measured by HRV. In a previous study

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from our group, no agreement between parasympathetic activity measured in the heart, pupil, and lungs were found (35), suggesting that parasympathetic activity measurements depend on the target organ. Yet a strong correlation between the bronchodilating effect of inhaled ipratropium bromide on exercise-induced bronchoconstriction (EIB) blocking efferent cholinergic pathways in the airways, and cardiac vagal activity, measured during a 4-s exercise test, is found in both healthy cross-country runners (20) and asthmatic children (21).

The previously cited studies suggest that increased parasympathetic activity is involved in the pathogenesis of asthma and BHR in athletes. Thus, measuring parasympathetic activity in athletes may be of clinical importance and may improve the understanding of the development of asthma in this group. However, the measurement method of parasympathetic activity seems to be of importance. The primary aim of the present study was to assess the association between BHR to methacholine and parasympathetic activity measured by variation in heart rate and by pupil constriction. Secondarily, we aimed to compare BHR to methacholine and parasympathetic activity between healthy and asthmatic cross-country skiers and swimmers, as well as in nonathletes.

MATERIALS AND METHODS

Subjects and design. We recruited cross-country skiers and swimmers from sport clubs in the southeast part of Norway, as well as through the National Olympic Center in Oslo, Norway. Inclusion criteria were to compete on a high national or international level, to train more than 10 $h \cdot wk^{-1}$, and to be 16–35 yr old. Simultaneously, healthy nonathletes within the same age range, who trained less than 5 $h \cdot wk^{-1}$, were recruited as control subjects, mostly students from the Norwegian School of Sport Sciences and the University of Oslo, as well as local high schools.

All subjects attended the laboratory on 2 d, separated by >24 h, but no more than 3 wk. Parasympathetic activity was measured in standardized settings in both visits. In addition, measurements of fractional exhaled nitric oxide (FE_{NO}) and skin prick test were performed, followed by a methacholine bronchial challenge at the first visit. A modified Allergy Questionnaire for Athletes, developed and validated for screening allergic diseases in athletes (4), was administered to record past or present history of asthma, allergy, and exercise-induced asthmalike symptoms in relation to sport participation. On the second visit, measurements of body composition were conducted. All subjects were to refrain from exercise and caffeine on the same day, as well as to avoid food and drinks >2 h before each visit. The subjects had to be free from any acute respiratory disease, including upper viral infections, for the last 3 wk and to refrain from exercise on the same day before each visit. Inhaled short acting β_2-agonists were withheld for 8 h before each visit; inhaled long-acting β_2-agonists, theophylline, and leukotriene antagonists were withheld for the last 72 h; antihistamines were withheld for the last 7 d; and orally administered glucocorticosteroids were withheld for the last month. Inhaled corticosteroids were not to be used on the same day. Athletes were grouped on whether they had current asthma or not. Current asthma was defined as a doctor's diagnosis of asthma, combined with the presence of either current BHR to methacholine ($PD_{20met} \leq 8$ μmol) or the current use of asthma medication. Data collection occurred from September 2013 to September 2014, and subjects with known or suspected allergies were not tested during the pollen season. The study was approved by the Regional Committee for Medical and Health Research Ethics. All subjects gave their written informed consent for participation, and an additional signed consent was acquired by a parent or a guardian when subjects were younger than 18 yr.

Methods. Parasympathetic activity was assessed noninvasively in two target organs: 1) the heart and 2) the pupil. 1) A 4-s exercise test (4sET) analyzed the acute cardiac vagal response to exercise (2,3). Subjects were instructed to pedal as fast as possible on a cycle ergometer with no load, from the fourth to the eighth second of a maximal inspiratory apnea. HRV was recorded with a heart rate monitor (Polar Electro®, OY, Kempele, Finland). The ratio between the longest RR interval before exercise and the shortest RR interval during the exercise was identified as the cardiac vagal index (CVI), as defined by Araújo et al. (2). The highest CVI from two consecutive trials was used for analysis. 2) The autonomic regulation of the pupil was assessed by pupillometry, according to Felipe et al. (14). A portable infrared PLR-200™ pupillometer (NeurOptics Inc., Berkeley, CA) stimulated the eye with a light flash (180-nm peak wave light) and measured the initial diameter (mm) of the pupil and just at the peak of the (minimal) constriction. The percent pupil constriction and the average and maximum constriction velocity ($mm \cdot s^{-1}$) were also recorded. Pupil amplitude was calculated by subtracting the minimal diameter from the initial pupil diameter. The subjects spent 15 min in a semidark room to adjust to low lighting levels before measurement. One pupil light response curve to each eye was recorded for each subject, and mean values were used for further analyses. The parasympathetic activity tests were performed on two separate days, with the mean values used for statistical analyses, to minimize effects from day-to-day variability.

FE_{NO} was measured with a single-breath online technique and a chemiluminescence analyzer (EcoMedics AG, Duerten, Switzerland). Subjects inhaled NO-free air to total lung capacity and exhaled for 10 s through a mouthpiece against a target pressure of 20 cm H_2O (1). Mean values of three measurements with a <10% difference were used in the analysis.

Lung function was measured by maximal expiratory flow volume curves (MasterScreen Pneumo spirometer; CareFusion, Höchberg, Germany) according to current guidelines (26) and recorded as forced expiratory volume in 1 s (FEV_1), forced vital capacity (FVC), and forced expiratory flow in 25%–75% of FVC. Predicted spirometry values were defined according to Quanjer et al. (31).

Allergy skin prick test was performed using extracts of 10 common allergens (ALK-Abelló as, Hørsholm, Denmark): dog, cat and horse dander, birch, timothy and mugwort pollens, mold (*Cladosporium herbarum*), house dust mite (*Dermatophagoides pteronyssinus*), cow's milk, and hen's egg white. A test was considered positive if at least one allergen caused a weal of ≥3 mm greater than the negative saline control in diameter, in the presence of a positive histamine control (6).

Methacholine bronchial challenge was performed during controlled tidal breathing with an inspiration-triggered Aerosol Provocation System (Jäger, Würzburg, Germany) (12). After inhalation of 0.9% isotonic saline, spirometry was performed to establish baseline FEV_1. Then subjects inhaled doubling doses of methacholine chloride (32 $mg \cdot mL^{-1}$) starting with 0.25 μmol. Spirometry was repeated 1 min after every delivered dose until FEV_1 decreased 20% from baseline (PD_{20met}), or the maximal dose of methacholine (24.48 μmol) was reached. A positive response was defined as a 20% reduction in FEV_1 at a cumulative dose of ≤2 μmol (0.4 mg) of methacholine, calculated by linear interpolation on the dose–response curve. Clinical significant BHR was defined as PD_{20met} ≤8 μmol (1.6 mg). We are aware that other groups have used a stricter cutoff of 4 or 2 μmol, and therefore we also analyzed our data using these criteria (10). After the methacholine provocation, all subjects received salbutamol inhalation (0.1 $mg \cdot mL^{-1}$ × 10 kg body mass) to reverse bronchial obstruction.

Body composition was measured by bioelectrical impedance analysis using Inbody 720 (Biospace Co. Ltd., Soul, Korea). The standardization of procedures included 5 min of resting and a minimum of 2-h fasting before measurement. To increase the reliability, the measurements were conducted twice (19), and the mean values were used in the analyses.

Statistical analyses. Continuous data are presented using standard summary statistics (mean and SD). Categorical variables are presented as counts (N) with percentages. Results are presented as mean values and β coefficients with 95% confidence intervals (CI), unless otherwise stated. Subjects with a PD_{20met} of >24.48 μmol were assigned a PD_{20met} value of 25 μmol, and subjects with a PD_{20met} of <0.1 μmol were assigned a PD_{20met} value of 0.1 μmol. Associations of BHR and parasympathetic activity were assessed by robust regression models with PD_{20met} as the dependent variable. For all parasympathetic variables, robust regression analyses were used, as the residuals were clearly nonnormal. We performed Hosmer's step-down procedure (18) retaining significant background variables only. We had the following basic set of background variables: age, sport type, and gender. Group means were compared using Student's t-test for two independent samples and ANOVA for three or more groups after tests for normality. *Post hoc* tests (Tukey's multiple comparisons technique) were applied to determine within-group differences. Chi-square tests (χ^2) were used to assess group differences of categorical variables. Correlations were calculated by Spearman's rank order correlation (ρ), or Pearson's correlation coefficient (r_p) where applicable. All P values less than 0.05 (5%) were considered significant. Analyses were conducted in IBM SPSS Statistics version 21.0 (SPSS Inc., Chicago, IL) and SAS version 9.4 (SAS Institute Inc., Cary, NC). Power calculations are based on variations in the mean values (represented by SD) from a study previously performed at our lab (35). A total sample of 90 subjects (30 in each group) achieves 80% power to detect a difference in percent pupil constriction and CVI between controls and the two athletes groups of 0.18 and 0.14, respectively, and the difference between two athlete groups of at least 0.06 for both variables.

RESULTS

Twenty-eight cross-country skiers (♂18/♀10), 29 swimmers (♂17/♀12), and 30 healthy nonathletes (♂14/♀16) completed both visits. Fourteen swimmers (48%) and 16 cross-country skiers (57%) met the criteria set for current asthma. Of these, seven athletes (five swimmers and two cross-country skiers) were not aware of asthma when entering the study. Demographic and clinical characteristics of the subjects are presented in Table 1. The occurrence of BHR (defined as PD_{20met} ≤2, ≤4, or ≤8 μmol) did not differ significantly between the asthmatic athletes, healthy athletes, and controls.

Parasympathetic activity and BHR. CVI was significantly associated with PD_{20met}, with the model explaining 18.2% (r^2) of the variation in PD_{20met} after adjusting for age, sex, and type of sport (Table 2). When adjusted for the type of sport, a stronger association between CVI and PD_{20met} was observed in swimmers as compared with the reference group (controls), but the association for cross-country skiers was not statistically significant. Neither of the pupillometry variables were associated with PD_{20met} in the crude analysis. However, when adjusting for age, sex, and type of sport, all parasympathetic pupillometry variables were significantly associated with PD_{20met} in swimmers (Table 2). Furthermore, the associations between PD_{20met} and any parasympathetic

TABLE 1. Demographic and clinical characteristics of the subjects given as mean ± SD.

	Athletes			Control subjects
	Asthma (n = 30)	Healthy (n = 27)	All (N = 57)	(n = 30)
Sex (♂:♀)	17:12	18:10	35:22	14:16
Weight (kg)	71.9 ± 11.7	72.1 ± 9.1	72.0 ± 10.5	72.0 ± 12.1
Muscle mass (%)	**48.8 ± 3.1***	**48.5 ± 6.8***	**48.7 ± 5.3***	44.3 ± 4.8
Fat mass (%)	**14.0 ± 4.7***	**13.5 ± 6.0***	**13.7 ± 5.4***	21.0 ± 7.3
FEV_1 (% predicted)	104.6 ± 13.5	106.2 ± 10.1	**105.4 ± 12.0***	99.8 ± 10.4
FVC (% predicted)	**112.5 ± 12.0***	110.2 ± 11.7	**111.4 ± 11.8***	104.3 ± 11.2
FE_{NO} (ppb)	19.8 ± 11.4	17.6 ± 11.2	18.7 ± 11.2	15.0 ± 6.5
Atopy (%)	76.4%	47.4%	61.1%	51.6%
BHR (PD_{20met} <8μmol, %)	62.5%	51.7%	58.3%	38.7%
Training hours per week	17.9 ± 4.4	17.3 ± 5.3	17.6 ± 4.8	NA

Atopy is defined as a positive skin prick test to more than one allergen.
FE_{NO}, fractional exhaled nitric oxide; PD_{20met}, methacholine provocation dose causing >20% reduction in FEV_1; NA, not analyzed.
*Significantly different from controls (P < 0.05).
Significant results are shown in bold.

TABLE 2. Associations (β coefficients with 95% CI) of BHR to methacholine (PD_{20met}, dependent variable) with parasympathetic activity variables measured by pupillometry and HRV (independent variables) in competitive swimmers (n = 29), cross-country skiers (n = 28), and 30 healthy nonathletes (reference group).

Variable	β (95% CI)	r^2
Pupillometry		
Pupil constriction		
Crude	0.16 (−0.27 to 0.59)	0.007
Adjusted model[a]	2.86 (−1.18 to 6.89)	0.110
Adjusted model[b]	2.98 (−0.83 to 6.80)	0.219
Swimming[c]	**−9.39 (−15.40 to −3.37)***	
Cross-country skiing[c]	−4.79 (−10.19 to 0.60)	
Pupil amplitude		
Crude	−0.35 (−8.30 to 7.60)	0.000
Adjusted model[a]	0.57 (−7.12 to 8.28)	0.102
Adjusted model[b]	3.26 (−0.65 to 7.17)	0.180
Swimming[c]	**−8.63 (−14.83 to −2.44)***	
Cross-country skiing[c]	−3.88 (−9.24 to 1.51)	
ACV		
Crude	−3.10 (−7.43 to 1.14)	0.024
Adjusted model[a]	−2.98 (−7.15 to 1.19)	0.110
Adjusted model[b]	3.65 (−0.27 to 7.57)	0.195
Swimming[c]	**−8.24 (−14.38 to −2.10)***	
Cross-country skiing[c]	−3.87 (−9.24 to 1.51)	
MCV		
Crude	−2.92 (−6.25 to 1.14)	0.034
Adjusted model[a]	3.47 (−0.52 to 7.46)	0.130
Adjusted model[b]	3.57 (−0.34 to 7.48)	0.194
Swimming[c]	**−8.04 (−13.17 to −1.91)***	
Cross-country skiing[c]	−3.57 (−0.34 to 7.48)	
HRV		
CVI		
Crude	**−19.67 (−29.26 to −2.09)***	0.057
Adjusted model[a]	**0.55 (0.21 to 0.88)***	0.086
Adjusted model[b]	**−13.88 (−26.77 to −0.99)***	0.182
Swimming[c]	**−8.32 (−13.03 to −3.61)***	
Cross-country skiing[c]	−3.09 (−7.70 to 1.51)	

MCV, maximal constriction velocity; AVC, average constriction velocity.
[a]Adjusted for age and sex.
[b]Adjusted for age, sex, and type of sport.
[c]Additional effect from being a swimmer or cross-country skiers, respectively, as compared with the reference group.
*P value <0.05. Significant results are shown in bold.

variables were not significantly different when only including the asthmatic athletes, or subjects with severe or clinically relevant BHR ($PD_{20met} \leq 2$, 4, or 8 μmol), respectively (data not presented). No correlations (ρ) were observed between CVI and any of the pupillometry variables (data not presented).

Differences between healthy and asthmatic athletes. Athletes with asthma showed increased parasympathetic activity, in terms of percent pupil constriction (P = 0.002), as compared with healthy athletes (Table 3). However, control subjects also showed increased pupil constriction as compared with healthy athletes (P < 0.001). Healthy athletes had increased initial and minimal pupil diameter as compared with both asthmatic athletes (P < 0.01) and control subjects (P < 0.01). Other parasympathetic variables from pupillometry or CVI did not differ between asthmatic athletes, healthy athletes, or controls. We found no differences in neither of the parasympathetic activity parameters between subjects grouped by PD_{20met} cutoff values of 2, 8, and 16 μmol (data not presented). No differences in BHR occurrence or pupillometry parameters were found between athletes using inhaled corticosteroids and athletes who did not (data not presented). No statistical difference in FE_{NO} was found between asthmatic and healthy cross-country skiers (Table 1). Although not significant, increased CVI were observed in athletes as compared with controls.

Differences between swimmers and cross-country skiers. The distribution of PD_{20met}, as shown by the Kaplan–Meier curves (Fig. 1), differed significantly between swimmers, cross-country skiers, and controls (P < 0.05). Fourteen swimmers (48%) had a severe BHR ($PD_{20met} \leq 2$ μmol) as compared with only one cross-country skier (P < 0.001), and 72% swimmers had clinical BHR ($PD_{20met} \leq 8$ μmol) as compared with 44% of the cross-country skiers and 39% controls (P = 0.015) (Table 4). The swimmers showed an increased mean FVC compared with cross-country skiers (P = 0.009). A weak to moderate inverse correlation was found between FVC and PD_{20met} (ρ = −0.22, P = 0.036). The swimmers were younger than the cross-country skiers (P < 0.001) and trained more weekly hours than the cross-country skiers (P < 0.001). PD_{20met} did not correlate with training hours per week (ρ = −0.25, P = 0.08). Furthermore, cross-country skiers had increased FE_{NO} as compared with the swimmers (P = 0.009).

Variations in parasympathetic activity. Mean day-to-day variance for pupillometry parasympathetic parameters ranged from 0.3% to 3.2%. Group means of initial pupil diameter (P = 0.002) and minimum pupil diameter (P = 0.02) were increased on day 2. Measurements of CVI did not differ between days (P = 0.233). The mean day-to-day CVI variance was 3.4%. The mean difference between day 1 and day 2 was similar when comparing subjects who tested on the same time of day (morning, midday, or afternoon) on both days with subjects who tested at different time points. The majority of the tests (59.5%) were performed in the morning (6:00–10:00 a.m.). No differences in variation between day 1 and day 2 were found between subjects where measurements were performed at the same time of day both

TABLE 3. Parasympathetic activity variables, presented as mean (95% CI), of pupillometry, and HRV measured at the onset of exercise by the 4sET in competitive athletes with and without asthma, and in healthy nonathletes (controls).

Method	Variable	Asthmatic athletes (n = 30)	Healthy athletes n = 27)	Controls (n = 30)
Pupillometry	Initial diameter (mm)	6.53 (6.31–6.75)	**7.00 (6.82–7.18)***	6.43 (6.08–6.77)
	Minimal diameter (mm)	4.54 (4.32–4.76)	**5.10 (4.89–5.30)***	4.41 (4.10–4.72)
	Pupil amplitude (mm)	1.99 (1.89–2.09)	1.90 (1.84–1.97)	2.02 (1.91–2.13)
	Pupil constriction (%)	30.7 (29.1–32.3)	**27.4 (26.1–28.7)***	32.1 (30.3–33.9)
	MCV ($mm \cdot s^{-1}$)	5.38 (5.15–5.63)	5.29 (5.12–5.47)	5.61 (5.35–5.88)
	ACV ($mm \cdot s^{-1}$)	4.10 (3.92–4.27)	3.91 (3.77–5.05)	4.18 (3.99–4.38)
4sET (HRV)	CVI	1.44 (1.38–1.49)	1.41 (1.35–1.46)	1.38 (1.32–1.44)

MCV, maximal constriction velocity; AVC, average constriction velocity.
*P < 0.05 (ANOVA with Tukey's HDS *post hoc* test). Significant results are shown in bold.

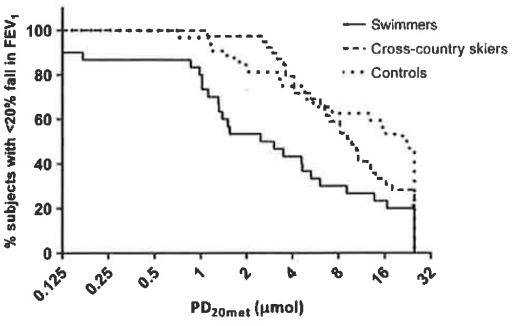

FIGURE 1—Kaplan–Meier plot showing the distribution of the provocation dose of accumulated inhaled methacholine (PD_{20met}) causing a ≥20% reduction in FEV_1 in cross-country skiers and swimmers and healthy nonathletes (controls). All Kaplan–Meier curves are significantly different from one another ($P = 0.005$).

days (64% of all subjects) as compared with subjects where time of day varied from day 1 to day 2.

We found no significant differences between left and right eye for any of the pupillometry variables, and significant correlations (r_p = 0.62–0.89) were found between the eyes ($P < 0.03$). Moderate to strong correlations (r_p = 0.58–0.90, $P < 0.001$) were observed in all pupillometry parameters when comparing day 1 and day 2.

DISCUSSION

The findings from the present study showed that the association between BHR and parasympathetic activity depends on the measurement procedure or target organ of parasympathetic activity. Furthermore, the type of sport influences these associations. If the same applies to other sports or to indirect measurement procedures of BHR remains to be clarified.

A negative association of CVI to PD_{20met} was found, in particular in swimmers, demonstrating that a higher CVI was related to more severe BHR. This supports the hypothesis that increased parasympathetic activity is related to more severe BHR in these athletes and corroborates to previous results (11,22,23,34). However, the same was not found regarding pupillometry variables. Bronchoconstriction is mediated, such as bradycardia, by afferent nerves in the nervus vagus, allowing a link between the regulation of the heart and the bronchi. This may explain why PD_{20met} was associated with CVI and not with pupillometry, and the model explained 18% of the variation in PD_{20met}. In a previous study from our group (35), we found no associations between CVI, nor pupillometry variables, to the reversibility to inhaled ipratropium bromide, an anticholinergic bronchodilator. Furthermore, Horváth et al. (17) found no agreement between HRV and resting specific airway resistance, which they suggested to represent the vagal activity of the bronchi. These findings may reflect that although the parasympathetic regulation of the heart and the bronchi is mediated through nervus vagus, neurogenic differences exist between these organs, for instance, regarding neural pathways, receptor sensitivity, or regulation of sympathetic–vagal balance. In addition, the parasympathetic bronchial tone as shown by reversibility to inhaled ipratropium bromide, specific airway resistance, or cholinergic sensitivity to inhaled methacholine may reflect different aspects of the parasympathetic regulation of the bronchi. The results from the present study suggest that in regard to parasympathetic activity assessments in athletes, the heart is a more appropriate target organ as compared with the pupil. Uusitalo et al. (37) recommend time domain and frequency domain indices of HRV measurements for the assessment of parasympathetic activity in athletes. The HRV protocol used in the present study, the 4sET, is found to be comparable with these HRV indices in healthy subjects (28).

When adjusted for the type of sport, differences in the associations of parasympathetic parameters to PD_{20met} became apparent, with the associations being significant in swimmers and not in cross-country skiers. Similarly, a correlation between BHR to methacholine and parasympathetic parameters of pupillometry is previously reported in swimmers

TABLE 4. Clinical characteristics, presented as mean (95% CI), of the athletes (stratified by sport) and controls.

	Athletes			Controls
	Swimmers (♂17: ♀12)	Cross-country skiers (♂18: ♀10)	All (♂35: ♀22)	(♂14: ♀16)
FEV_1 (% predicted)	**107.5 (102.8–112.2)***	104.5 (100.6–108.3)	**105.4 (102.3–108.4)***	99.8 (95.9–103.7)
FVC (% predicted)	**115.7 (111.1–120.2)***	108.5 (105.1–112.0)	**111.4 (108.4–114.4)***	104.3 (100.1–108.5)
FE_{NO} (ppb)	**14.7 (12.4–17.2)†**	**22.2 (17.1–27.4)***	18.7 (15.6–21.7)	15.0 (12.4–17.6)
Atopy (%)	62.1%	62.1%	62.0%	51.6%
PD_{20met} <8 μmol (%)	**72.4%*,†**	42.3%*	57.4%	38.7%
PD_{20met} <4 μmol (%)	**58.6%*,†**	21.1%	37.7%	25.8%
PD_{20met} <2 μmol (%)	**48.3%*,†**	2.6%	24.6%	16.1%
Drug use (%)				
Inhaled corticosteroids	15.4%	**28.6%***	22.2%	9.0%
Inhaled bronchodilator	**23.1%***	**25.0%***	**24.0%***	8.0%
Baseline HR (bpm)	60.2 (56.7–63.7)	**57.7 (53.8–61.6)***	**58.9 (56.3–61.5)***	64.8 (60.8–68.8)
Training hours per week	**21.0 (19.2–22.8)†**	14.6 (13.4–15.9)	17.6 (16.2–19.0)	NA

Atopy is defined as a positive skin prick test to more than one allergen.
PD_{20met}, the methacholine provocation dose causing >20% reduction in FEV_1; NA, not analyzed.
*Significantly different from controls ($P < 0.05$).
†Significantly different from cross-country skiers ($P < 0.05$). Significant results are shown in bold.

with severe BHR (11). This may be explained by the type of training, training volume, or training environment. The exposure of chlorine derivate from indoor swimming pools may irritate the airways, and the high total volume of chlorine inhaled by competitive swimmers is considered responsible for the high occurrence of asthma and BHR reported in these athletes (5,13). Indeed, in the present study, more swimmers than cross-country skiers had a severe BHR (PD_{20met} <2 μmol). Thus, our results may show that the association between BHR and parasympathetic activity is dependent on BHR severity and may be related to the environmental exposure of the swimmers. This means that the use of inhaled corticosteroids, which may influence BHR severity, may have potentially confounded our results. Thus, the differences observed between the type of sport may be influenced by BHR severity and use of inhaled corticosteroids. Five swimmers and two cross-country skiers were unaware of asthma when entering the study and were thus untreated. However, when adjusted for BHR at different PD_{20met} cutoff points (2, 4, or 8 μmol), no differences in parasympathetic activity were observed, nor a correlation between parasympathetic activity and PD_{20met} as opposed to the results of Couto et al. (11). Furthermore, no differences in parasympathetic activity were found between athletes using inhaled corticosteroids and athletes who did not.

A negative correlation between PD_{20met} and age in cross-country skiers was previously reported (34), suggesting that years of accumulated training will increase the risk of BHR in athletes. Pedersen et al. (29) found a lower BHR prevalence in swimmers age 12–16 yr as compared with a control group of unselected adolescences. Yet despite being younger, the swimmers in the present study had higher occurrence of BHR as compared with the (older) cross-country skiers. The 72% prevalence of clinical BHR among swimmers in our study agrees closely with the results of previous studies (5,22,39). Martin et al. (25) reported the same BHR percentage measured by eucapnic voluntary hyperpnoea tests in pool-based athletes. In line with previous studies (5,25,29), the swimmers of the present study had higher lung volumes compared with control subjects and cross-country skiers. Although a weak but significant correlation between FVC and BHR to methacholine (PD_{20met}) was found, it is uncertain if the increased lung volumes found in swimmers are significantly associated with BHR.

We found that asthmatic athletes had increased pupil constriction compared with healthy athletes, but no differences were found in the other pupillometry parameters. An unexpected finding, which may confuse our interpretations, is that increased pupil constriction was also found in healthy nonathletes. Likewise, Filipe et al. (14) did not observe differences in pupillometry parameters between athletes and nonathletes. However, when stratified by the type of sport, endurance-trained runners showed increased pupil amplitude and percent pupil constriction as compared with soccer players, swimmers, and gymnasts, as well as to sedentary control subjects. As opposed to Couto et al. (11), we did not observe differences in pupillometry parameters between subjects when grouped according to their PD_{20met}. Furthermore, increased initial and minimal pupil diameters were found in healthy athletes, which is proposed to reflect sympathetic–parasympathetic balance (14), and the differences observed in pupil constriction and amplitude in cross-country skiers, or in healthy athletes compared with other groups, must therefore be interpreted with care. Surprisingly, no difference in CVI was observed between controls and athletes in the present study. This is in contrast to previous studies reporting increased cardiac vagal tone in endurance athletes compared with nonathletes as well as the positive relationship to $\dot{V}O_{2max}$ (7,16). As power calculations were performed, and the number of subjects included in the present study was similar or greater than that of previous studies showing significant results (11,14,20), a power problem seems unlikely. The controls did exercise regularly, and many of them reported that they previously had participated in competitive sports. However, it is unsure if the inclusion of more sedentary subjects would obtain significant results. Nevertheless, a trend toward a higher CVI in both swimmers and cross-country skiers compared with controls was seen, and the athletes showed a decreased baseline heart rate as compared with the controls, which is suggestive of a higher vagal cardiac flow. In a study by Araújo et al. (2), no difference in parasympathetic activity between athletes (n = 90) and controls (n = 58) was found using the 4sET. This is in agreement with our results and suggest that the 4sET is not sensitive enough to detect differences between such groups. Unfortunately, no objective tests for physical fitness or performance levels are available in the present study. Therefore, we can only assume that the athletes had higher $\dot{V}O_{2max}$ than the control subjects based on the inclusion and exclusion criteria, and indicated by the increased muscle mass and decreased body fat shown in athletes as compared with controls.

The methacholine provocation challenge is a well-known direct test used for the assessment of BHR in asthmatics (12) and is regarded as a more sensitive test as compared with indirect tests such as exercise tests, or Mannitol challenge (12,36), but similar sensitivity is reported regarding the eucapnic voluntary hyperventilation test (33). However, the PD_{20met} may also reflect parasympathetic bronchial tone by sensitivity to methacholine. Methacholine is an ester of acetylcholine (ACh), a known transmitter substance of the parasympathetic nervous system, with the parasympathetic (vagal) nerves innervating the contractive muscles of the bronchi. Therefore, one can argue that the increased sensitivity to methacholine, defined as PD_{20met}, is reflecting the parasympathetic bronchial tone. This can, at least partly, explain the high BHR prevalence observed in asthmatic endurance athletes. It is also of interest that it has been described how ACh is produced in an inflamed respiratory mucosa (38), suggesting that ACh may have an additional nonneural role in the pathogenesis of asthma. On the basis of a study from our group (34), reversibility tests to the inhaled

anticholinergic ipratropium bromide may be of value for the determination of bronchial parasympathetic tone and asthma treatment strategies for athletes.

A limitation of the present study is the cross-sectional design. Thus, we cannot determine causality in the associations of parasympathetic activity, BHR, and endurance training in these athletes. An unexpectedly high occurrence of BHR was observed in the controls, which may be caused by a selection bias. The use of anti-asthmatic drugs could influence BHR and/or parasympathetic activity measurements. However, these drugs were withheld >8 h before the tests. Inhaled corticosteroids were not withheld because of ethical considerations for the competing athletes, which is a limitation of the present study. When comparing the athletes who used inhaled corticosteroids or bronchodilators with nonusers, we found no differences in neither of the parasympathetic activity parameters. However, small sample sizes and lack of power may influence these results and they must therefore be interpreted carefully.

In the present study, we used the Polar® heart rate monitor to assess HRV, which is shown to be comparable with ECG (32). A previous study from our group showed good repeatability for the 4sET and pupillometry (35). A strength of the present study is that parasympathetic activity was measured in two target organs, as well as on two different days. In the present study, we aimed to schedule all visits at the same time of the day to avoid potential circadian variations. However, because of practical reasons, the time of testing could vary, but the day-to-day variability was low, and no effect from diurnal variation seemed to influence our results. All parameters of parasympathetic activity are performed at rest, except CVI, which was assessed during a short (4-s) cycling exercise. Thus, differences of autonomic regulation during exercise may differ from our observations.

CONCLUSION

The results from the present study show that the association between BHR and parasympathetic activity differ between measurement procedures of parasympathetic activity. Cardiac vagal activity is associated with BHR to methacholine, but not pupillometry parameters, suggesting that the parasympathetic activities of the heart and lungs are more closely related than to the activity of the pupils. However, the associations between parasympathetic activity and PD_{20met} are dependent of the type of sport (swimming or cross-country skiing) and may thus be influenced by training environment or other sport-specific factors such as type of training. More severe BHR is apparent in swimmers as compared with cross-country skiers.

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Paper II

The role of airway inflammation and bronchial hyperresponsiveness in athlete's asthma

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Word count: 3998

ABSTRACT

PURPOSE Asthma is frequently reported in endurance athletes. The aim of the present study was to assess the long-term airway inflammatory response to endurance exercise in high-level athletes with and without asthma.

METHODS In a cross-sectional design, 20 asthmatic athletes (10 swimmers, 10 cross-country skiers), 19 athletes without asthma (10 swimmers, 9 cross-country skiers) and 24 healthy non-athletes completed methacholine bronchial challenge, lung function tests and sputum induction on two separate days. All athletes competed on a national or international level and exercised ≥10 hours/week. The non-athletes exercised ≤5 hours/week and reported no previous lung disease. Bronchial hyperresponsiveness (BHR) was defined as a methacholine provocation dose causing 20% decrease (PD_{20met}) in the forced expiratory volume in one second (FEV_1) of ≤8 μmol.

RESULTS BHR was present in 13 asthmatic athletes (62%), 11 healthy athletes (58%) and eight healthy non-athletes (32%) and the prevalence differed among groups (p=0.005). Sputum inflammatory and epithelial cell counts did not differ between groups and were within the normal range. Median (25^{th} to 75^{th} percentiles) sputum interleukin (IL)-8 was elevated in both asthmatic (378.4 [167.0, 1123.4]) and healthy (340.2 [175.5, 892.4]) athletes as compared to healthy non-athletes (216.6 [129.5, 314.0], p=0.02). No correlations were found between PD_{20met} and sputum cell counts.

CONCLUSION Independent of asthma diagnosis, a high occurrence of BHR and increased sputum IL-8 were found in athletes as compared to non-athletes. Airway inflammation or epithelial damage were not related to BHR.

INTRODUCTION

Asthma in athletes is frequently observed (1), and the clinical characteristics seem to differ from those observed among non-athlete asthmatics. For instance, exercise-induced respiratory symptoms are frequently reported among athletes, yet no associations to objective clinical findings are apparent (2, 3). In fact, a distinct phenotype of "sport asthma" has recently been reported (4).

Bronchial hyper responsiveness (BHR) is a well-known characteristic of asthma (5). While swimmers and cold air endurance athletes do have increased BHR when compared to healthy non-athletes (6, 7), there is no difference when comparing to asthmatic individuals (7). Interestingly, while swimmers have increased lung function compared to both non-athletes as well as athletes of other sports, they have also shown a large prevalence of BHR (3, 6-8). The mechanisms of asthma in athletes are reportedly related to the accumulated strain from high ventilation rates upon the airways, in combination with unfavorable environmental exposures, such as inhalation of cold and dry air or chlorine-derivate of indoor swimming pools (9). In addition, increased parasympathetic activity due to systematic endurance exercise is suggested to influence bronchial tone and thus BHR in endurance-trained athletes (6). Bronchial epithelial damage is proposed to be an important feature of athlete's asthma (10) and increased sputum epithelial cells are shown in athletes as compared to both asthmatic and healthy non-athletes (3, 7, 11), as well as acutely post exercise (12).

The role of airway inflammation in athlete's asthma is not fully accounted for, and evidences of both acute and long-term inflammatory effects of exercise are conflicting. Some studies have shown an increased neutrophilic airway inflammation in athletes within different sport disciplines (3, 11-15), while other studies show minimal or no airway inflammation (2, 7, 8, 16). However, several studies report increased inflammatory mediators in plasma or sputum, such as CC16 (3, 17, 18), IL-8 (19), IL-1β and IL-6 (3, 19). Notably, neither of these studies have stratified athletes by asthma diagnosis and it is not clear if the airway inflammatory response to systematic endurance exercise is similar in athletes with and without asthma. Furthermore, there are gaps in the understanding of the long-term response to exercise regarding the role of airway inflammation and its relation to BHR. The aim of the present study was to assess the long-term effect of systematic endurance exercise upon airway inflammation and BHR in high-level asthmatic and non-asthmatic athletes within sports known to be of high-risk for asthma, namely swimming and cross-country skiing (4, 9). In addition, we wanted to examine the relationship between airway inflammation and BHR in these athletes.

MATERIALS AND METHODS

Subjects and design

In the present cross-sectional study, one group of athletes with a previous asthma diagnosis (n=27, 13 swimmers and 14 cross-country skiers), one group of athletes without doctor diagnosed asthma (n=26, 13 swimmers and 13 cross-country skiers) and one group of healthy non-athletes (n=27) completed methacholine bronchial challenge, lung function tests and sputum induction. Athletes were grouped on whether they had current asthma or not. Current asthma was defined as a doctor's diagnosis of asthma, combined with the presence of either current BHR to methacholine ($PD_{20met} \leq 8$ μmol) or the current use of asthma medication. Only the subjects with eligible sputum samples (as described in induced sputum section) were included in the present study. The final study population consisted of 20 athletes with asthma (10 swimmers), 19 healthy athletes (10 swimmers) and 24 healthy non-athletes. All subjects were non-smokers, aged 16-35 years, and both men and women were included.

Athletes were recruited from regional sport clubs, as well as through the National Olympic Center in Oslo, Norway. Control subjects were recruited from the Norwegian School of Sport Sciences (NSSS), University of Oslo and from local high schools through online advertisements on social media channels. Inclusion criteria for athletes were competition at high national or international levels and more than 10 hours (h) of exercise per week. Control subjects were not to take part in competitive sports and not to exercise more than 5 h per week.

Data collection was carried out from September 2013 to September 2014. The subjects with known or suspected allergies were not tested during the pollen season. Inhaled short acting β_2-agonists were withheld for eight hours before testing; inhaled long-acting β_2-agonists, oral theophylline, and leukotriene antagonists were withheld for the last 72 hours; antihistamines were withheld for the last 7 days; and orally administered glucocorticosteroids were withheld for the last month. Inhaled corticosteroids were not to be used on the day of testing (20). The subjects had to be free from any acute respiratory illness for the last three weeks and refrain from exercise on the day of testing (>12 hours). All subjects attended the laboratory at NSSS on two different visits, separated by < 3 weeks and >24 hours. At the first visit measurements of fractioned exhaled nitric oxide (FE_{NO}), spirometry, and skin prick test (SPT) followed by a methacholine bronchial challenge was performed. On the second visit, blood sample was collected and induced sputum induction was carried out. A questionnaire was administered to document the subjects' past or present history of asthma and allergy (21). All subjects gave their written informed consent for participation and an additional signed consent was acquired by parent or guardian for subjects were under

the age of 18 years. The present study was approved by the Regional Committee for Medical and Health Research Ethics (2013/167).

Test protocols

Fractional exhaled nitric oxide (FE_{NO}) was measured with a single-breath online technique at a constant expiratory flow rate of 50 $ml \cdot s^{-1}$ in accordance to the manufactures instructions (EcoMedics AG, Duerten, Switzerland) (22). Mean values of three measurements with a <10% difference were used in the analysis.

Lung function was measured by maximal expiratory flow volume curves (MasterScreen Pneumo Jäger ®, Würzburg, Germany) according to current guidelines (23), and recorded as forced expiratory volume in one second (FEV_1), forced vital capacity (FVC) and forced expiratory flow at 25-75% of FVC (FEF_{25-75}). Predicted spirometry values were defined according to Quanjer et al. (24).

Allergy skin prick test was carried out with extracts of ten common allergens (ALK-Abelló as, Hørsholm, Denmark): dog, cat, horse dander, birch, timothy, mugwort pollens, mold (Cladosporium herbarium), house dust mite (Dermatophagoides pternoyssinus), cow's milk and hen's egg white. A subject was classified as atopic if at least one allergen caused a weal of ≥3 mm in diameter greater than the negative control, in the presence of a negative saline control and a positive histamine (25).

Methacholine provocation challenge was performed, using an inspiration-triggered Aerosol Provocation System (APS) Jäger nebulizer (Würzburg, Germany), according to guidelines of the American Thoracic Society (26). After baseline measurement of lung function, subjects inhaled doubling doses of methacholine chloride (32 $mg \cdot mL^{-1}$) from a starting dose of 0.25 µmol and until a fall in FEV_1 of ≥20% (PD_{20met}) or if the maximal dose of methacholine (24.48 µmol or 4.8 mg) was reached. A subject was considered to have clinical BHR if their methacholine PD_{20} was < 8 µmol (1.6 mg).

Induced sputum was collected and processed as described by Alexis et al. (27). All subjects were pretreated with inhaled salbutamol (0.1 $mg \cdot ml^{-1} \cdot 10$ kg body $mass^{-1}$) mixed in 1 ml isotonic NaCl before the sputum induction. Subjects inhaled 3% (w/V), 4% and 5% hypertonic saline for 7 min via an ultrasonic nebulizer (DeVilbiss Healthcare Ltd., West Midlands, UK), respectively. After each inhalation, the subjects were asked to blow their nose, rinse their mouth, and perform a chesty-type cough. Expectorate was collected into a sterile container and lung function tests were repeated. Sputum was processed within 2 hours after induction. Mucus plugs were selected from saliva and weighed and dissolved in phosphate buffered saline (PBS, Dulbecco's PBS Invitrogen, Burlington, ON, Canada) containing 0.1% (w/V) dithiothreitol (DTT) (Sigma, St.Louis, MO). The sample was mixed for 15 minutes, washed with PBS, filtered through a 48-µm pore mesh filter (Sintab, Oxie, Sweden) and centrifuged. Supernatants were frozen at -80°C. Total cell count and cell viability was determined with a

Bürker chamber using the trypan blue (0.4%) (Sigma) exclusion method. Calculation of cell differentiation was done on blinded cyto-centrifuged preparations stained with Diff-Quik (Merz-Dade, Dudingen, Switzerland) expressed as percentage of total. At least 400 cells/slide were counted by two investigators. The sputum sample was considered adequate if it was contaminated by <50% squamous epithelial cells and/or >50% viability.

Protein analysis in blood plasma and induced sputum supernatant.

IL-1β and IL-8 were measured with a DuoSet ELISA kit obtained from R&D (Minneapolis, USA). The analyses were performed according to instructions from the manufacturers. The kits used in the analysis were tested for DTT. CC16 was measured using the Human Club Cell Protein ELISA kit (detection limit 46 pg/ml) from BioVendor (Modrice, Czech Republic) according to the manufacturers protocol.

Statistical analysis

Continuous data are presented as means with 95% confidence intervals (CI) after tests for normality, unless otherwise stated. Categorical variables are presented as counts (N) with percentages. Subjects with a PD_{20met} of >24.48 µmol were assigned a PD_{20met} of 25 µmol and subjects with PD_{20met} of <0.1 µmol were assigned a PD_{20met} of 0.1 µmol. One-way analysis of variance (ANOVA) or Kruskal-Wallis tests were used to compare the three groups after tests for normality on continuous data. Post hoc tests (Tukey's multiple comparisons technique) were applied to determine within-group differences on normally distributed data. Mann-Whitney U Test for Independent samples were used to compare two groups of non-normally distributed data. Chi square tests (χ^2) were used to assess group differences of categorical variables. Correlations were calculated by Spearman's rank order correlation (ρ). P-values below 0.05 were considered significant. Statistical analyses were performed using IBM SPSS Statistics version 21.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Characteristics of the subjects are presented in Table 1. The non-athlete group was older than both athlete groups ($p<0.001$). The asthmatic athletes and healthy athletes exercised the same amount of hours per week. However, swimmers (22.2 hours [20.8, 23.6] mean [95%CI]) exercised more than cross-country skiers did (14.3 hours [12.8, 15.8]) ($p<0.001$). No differences were observed in prevalence of atopy between the groups.

Bronchial hyperresponsiveness

Clinical BHR ($PD_{20met} \leq 8$ µmol) was found in 67% of the asthmatic athletes, 58% of the healthy athletes and in 33% of the non-athletes (Figure 1) ($p=0.005$). Post-hoc analyses revealed no difference in BHR prevalence between asthmatic and healthy athletes ($p=0.07$). However, severe BHR ($PD_{20met} \leq 2$µmol) was more frequent in swimmers (n=8) of which seven had asthma) compared to cross-country skiers (n=1, $p=0.05$) (Figure 2).

Sputum inflammatory and epithelial cell counts

Total sputum cell counts were similar among the three groups and no significant differences were observed when analyzing the different types of leukocytes by number or percentage (Table 2). All subjects had eosinophils ≤2% of total cell counts of bronchial epithelial cells and leukocytes. Bronchial epithelial cells in induced sputum varied from 1.2 to 2.0 per cent of total cells, with no significant differences between groups. No differences were observed when comparing the percentage of the different leukocytes in sputum between subjects with or without BHR (defined as PD_{20met} <8 µmol, <4 µmol or <2 µmol) (Table 3). Sputum inflammatory or epithelial cell counts did not correlate to PD_{20met} (data not presented). No significant correlations were observed between weekly hours of exercise or years of sport participation and sputum neutrophils or epithelial cells among the athletes. Non-atopic subjects (n=39) showed similar sputum cell counts as the 26 atopic subjects (9 asthmatic athletes, 6 healthy athletes and 11 non-athletes) (data not presented).

Airway inflammatory markers

Both athlete groups had increased sputum IL-8 as compared to non-athletes ($p=0.02$) (Figure 3). However, no significant differences were observed in sputum IL-1β between asthmatic athletes, healthy athletes or non-athletes (Table 2). Neither IL-1β nor IL-8 correlated with PD_{20met}. However, sputum neutrophils (%) correlated with both IL-1β ($\rho=0.389$, $p=0.002$) and IL-8 ($\rho=0.481$, $p<0.001$). No group differences in either sputum or plasma CC16 were observed (Table 2). Neither sputum nor plasma CC16 correlated with PD_{20met} or sputum inflammatory or epithelial cell counts or differed between subjects with different

PD_{20met} (Table 3)However, sputum CC16 correlated inversely to years of sport participation (ρ=-0.367, p=0.039) in the athletes. A weak correlation between sputum and plasma CC16 was observed (ρ=0.281, p=0.024).

Fractional exhaled nitric oxide (FE_{NO}) was significantly increased in athletes with asthma as compared to non-athletes (p=0.018), but not healthy athletes (Table 1). Furthermore, cross-country skiers had increased FE_{NO} (21.7 [15.9, 27.5]) as compared to swimmers (15.1 [12.0, 18.2]). No differences were observed between atopic (19.5 [15.6, 23.5]) and non-atopic subjects (15.0 [12.1, 17.9]). FE_{NO} correlated with sputum eosinophils (ρ=0.509 [p=0.026]).

Lung function

Athletes, both asthmatic and healthy, showed increased FVC (% pred. p=0.009) and FEV_1 (% pred. p<0.001) as compared to healthy non-athletes (Table 1). Furthermore, swimmers had increased FVC (124.0 % of predicted [117.3, 130.7]) as compared to cross-country skiers (115.3 % of predicted [109.7, 121.9]), p=0.02). No lung function variables correlated with weekly hours of exercise, years of sport participation, sputum inflammatory or epithelial cells, nor PD_{20met}.

Drug use

Eight of the twenty athletes with asthma reported regular use of inhaled bronchodilators (β_2-agonist or ipratropium bromide). Use of inhaled corticosteroids was reported in seven asthmatic athletes of which four had BHR (PD_{20met} <8 µmol). No differences were observed between athletes reporting use of inhaled corticosteroids compared to athletes who did not used inhaled corticosteroids regarding lung function (FEV_1 and FVC), BHR (PD_{20met}), leukocytes, epithelial cell counts or inflammatory markers in sputum or plasma (IL-1β, IL-8 or CC16). The use of antihistamines was reported in eight athletes with asthma, three healthy athletes and six healthy non-athletes. One healthy athlete and two healthy non-athletes with a history of allergy/rhinitis reported use of bronchodilators, but not during testing.

DISCUSSION

The main findings of the present study were the high occurrence of BHR to methacholine in both asthmatic and non-asthmatic swimmers and cross-country skiers as compared to healthy non-athletes. Yet, increased airway inflammatory cells were not observed in either group. However, we found increased level of sputum IL-8 among the athletes, independently of asthma diagnosis, as compared to healthy non-athletes. IL-8 correlated with neutrophils in induced sputum.

The proportion (differential) and the absolute number of sputum inflammatory cells counts did not differ between asthmatic athletes, non-asthmatic athletes and non-athletes. Our results are in agreement with similar studies showing no to minimal airway inflammation present in swimmers and cold weather athletes (7, 16) and suggest that that the potential acute inflammatory response to exercise is reversible or that the long-term effect of endurance exercise does not involve airway inflammation. However, in the present study we found increased levels of IL-8 among the athletes compared to healthy non-athletes, suggesting that systematic endurance exercise may induce an inflammatory response in the airways, independently of asthma diagnosis. In the present study, the proportion of sputum neutrophils correlated significantly to both supernatant IL-8 and IL-1β, yet the correlations were moderate. It is conceivable that the stress of intensive exercise or cold air exercise may cause unspecific damage of bronchial epithelium that is associated with increased secretion of IL-8 and influx of neutrophils (12, 27). Similarly, Belda and colleagues (13) found a mild neutrophilic inflammation in the airways of both asthmatic and non-asthmatic athletes practicing water sports. IL-8 is a chemoattractant, and we could have expected an increase in the neutrophil level in sputum in the athletes of the present study that reflected the IL-8 level. But no such differences were found. However, we found a correlation between the concentrations of IL-8 and the proportion of neutrophils cells. In sputum the proportion of newly and old recruited neutrophils differ (28). It is therefore possible that IL-8 is a more sensitive marker than proportion of neutrophils when studying the activity level of the inflammation process in the lung. Increased plasma IL-8 was found in swimmers with BHR after a swim ergometer sprint, but not in swimmers without BHR (19), which may suggest a relationship between IL-8 and BHR. Yet, despite a large prevalence of BHR in the current sample, we found no association to sputum concentrations of IL-8, and conversely increased IL-8 was found in athletes both with and without BHR. The role of IL-8 in athletes with asthma and BHR thus needs further studies.

Increased sputum bronchial epithelial cells are found after a half-marathon run in non-asthmatic subjects (12), as well as >12 hours after exercise in swimmers (but not cold air athletes)(7) and is suggested to reflect epithelial damage with subsequent shedding of epithelial cells into the airway lumen (7). Serum CC16 has been used as a marker for epithelial damage in relation to chlorine exposure (29) and urinary

CC16 is shown to increase after a swimming exercise (17) and after an EVH challenge in both athletes and non-athletes with and without BHR (18). In the present study, we found no increase in sputum epithelial cells or CC16 in plasma nor sputum is athletes as compared to none-athletes. Furthermore, no difference between asthmatic and non-asthmatic athletes were found. Possibly, our results may be related to the fact that the athletes in our study had not performed any exercise on the day of the sputum sampling. However, there are reports showing increased levels of serum CC16 in swimmers as compared to controls before exercise (3).

The presence of BHR with no increase in airway inflammatory cells is frequently found in endurance athletes (2, 7, 8). Although BHR is a feature of asthma and a majority of asthmatics have BHR, this state is not exclusive for asthma and may be present in healthy subjects as well (5). However, the large number of non-asthmatic athletes with BHR and increased plasma IL-8 in the present study may suggest undiagnosed asthma. At the same time, evidence of increased inflammatory mediators in sputum of non-asthmatic athletes with EIB is previously reported (30). In the present study, we set the methacholine cut-off for BHR at eight µmol (1.6 mg), a higher cut-off than commonly used as recommendation for medical treatment of asthma in athletes (31). However, this is a cut-off commonly used as cut-off for BHR in asthmatics (5). We also analyzed our data using stricter cut-offs of 4 or 2 µmol, which did not change our results (Table 3). In the present study, no correlations were found between PD_{20met} and sputum inflammatory cells, questioning the link between airway inflammation and BHR in athletes. Instead, it is conceivable that the BHR observed may be caused by delayed repair of airway epithelial damage (10), epithelial dysfunction (32) or increased parasympathetic tone (6). However, allergy, as measured by a SPT, was frequently observed among the asthmatic athletes, which suggest that mechanisms involving atopy could be involved in asthma pathogenesis in the athletes.

The differential sputum cell counts did not differ between the asthmatic and non-asthmatic swimmers and cross-country skiers (Table 2). However, the low number of subjects in each group limit the present study's power to disguise possible differences between types of sport. In line with previous studies (7, 8, 16), the swimmers of the present study had increased lung function compared to non-athletes and more severe BHR (<2 µmol) compared to cross-country skiers. FE_{NO} was increased in cross-country skiers as compared to swimmers. There were no differences in the occurrence of atopy between sport types. Six of 19 (32%) cross-country skiers and nine of 20 (45%) swimmers had a positive SPT. However, two cross-country skiers had a FE_{NO} >50 ppb, one of whom was allergic, which influence the mean in this group. In contrast to our results, Bougault and colleagues (2009) found a mild eosinophilic inflammation in swimmers, but not in cold-air athletes (including cross-country skiers), as compared to healthy control subjects. However, similar to our study, Martin and colleagues (2012) found no difference in sputum

eosinophils between swimming pool-based athletes and non-pool based athletes, despite a markedly higher incidence of BHR in the pool-based athletes. Notably, the swimmers in the present study exercised more weekly hours than the cross-country skiers did, yet, they were younger than the cross-country skiers and thus had accumulated fewer years with systematic exercise as active athletes. It has previously been found that both sputum eosinophils and neutrophils correlate to the amount of weekly exercise performed in swimmers and cold weather athletes, even though the degree of sputum inflammatory cells are not increased (7, 13). Such associations were not found in the present study. Inhalation of chlorine-derivate from indoor swimming pools may affect the airway epithelial layer that may make them more prone to methacholine or other substances that influence the smooth muscles surrounding the bronchi (10).

A strength of the present study was that we studied airway inflammation using induced sputum cells provided directly from the lower airways (33). We found increased levels of IL-8 among the athletes, but did not find any differences in sputum cells between the groups. However, the present study was not originally powered to detect differences in sputum inflammatory cells (6). In addition, our measurements were made >12 hours post exercise which may explain the lack of inflammatory cells found in sputum. This is a limitation of the current study, as both pre- and post-exercise samples would have allowed for a more complete assessment of the inflammatory response to exercise in athletes. Data collection was carried out throughout a year, including the competitive seasons for cross-country skiers (November-March) as well as for swimmers who compete throughout the year. Thus, recent competitions and training activity with high intensity, as well as seasonal variations, may influence BHR and airway inflammation (34). The results of the present study will not reflect post exercise conditions, but the general state of the airways in competitive swimmers and cross-country skiers who exercise >10 hours per week. However, our results may be affected by the use of inhaled corticosteroids in seven of the 20 asthmatic athletes, which may influence both inflammatory cell distribution and BHR (35). The non-athletes in the present study had sputum neutrophil and eosinophil levels comparable to low exposed or non-exposed healthy non-athletes in previous studies (7, 14).

Sputum is mainly collected from the central airways (28, 33), while exercise-induced bronchoconstriction (EIB) is known as a phenomenon that occurs in the peripheral airways (36). This may explain the lack of association between the sputum result and PD_{20met}. The use of impulse oscillometry (IOS) might have provided interesting insight into the bronchial response to methacholine as IOS is shown to be more sensitive than spirometry in detecting EIB in athletes after indirect provocations challenges. Thus, it might even detect additional cases of airway dysfunction in athletes (37, 38). A high proportion of healthy swimmers are shown to be positive to mannitol (39), suggesting that a mannitol test (or another indirect provocation challenge) performed in our individuals could have provided another access to the

inflammation, even though indirect tests as Mannitol bronchial provocation are usually less sensitive than direct tests such as methacholine bronchial challenge (5). The airway response to indirect as compared to direct bronchial provocation challenges may vary between subjects. This lack of agreement may reflect the different underlying mechanisms of BHR in the airways. As we did not include an indirect test in the present study, our results are limited to those athletes with a positive response to a methacholine bronchial challenge. Furthermore, it has been stated that methacholine bronchial provocation is more related to airway remodeling, being a direct challenge test for BHR as opposed to indirect tests, such as exercise tests or the mannitol or EVH test, which have been regarded as more related to airway inflammation (40).

CONCLUSION

The results from the present study show that the long-term response to systematic endurance exercise (as measured >12 h post exercise) in competitive swimmers and cross-country skiers is characterized by BHR and increased IL-8, but not increased airway inflammatory cells. Bronchial hyperresponsiveness is frequent in both asthmatic and non-asthmatic athletes as compared to healthy non-athletes and is not related to airway inflammation or sputum epithelial cells. Sputum IL-8 may be a marker of the long-term airway inflammatory response of systematic exercise among high-level swimmers and cross-country skiers.

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Table 1 Characteristics of athletes with asthma, healthy athletes and healthy non-athletes.

	Asthmatic athletes (n=20)	Healthy athletes (n=19)	Healthy non-athletes (n=24)
Sex (male:female)	13:7	14:5	11:13
Sport type (s:XC)	10:10	10:9	NA
Age, years	**20.3 (18.3, 22.3)***	**18.6 (17.6, 19.6)***	27.3 (24.9, 29.7)
FEV_1 (% of predicted)	**108.2 (103.3, 113.1)***	**106.4 (101.5, 111.4)***	97.6 (93.6, 101.6)
FVC, (% of predicted)	**115.0 (109.9, 120.1)***	**110.2 (103.8, 116.6)***	102.0 (97.6, 106.3)
Training hours /week	18.2 (16.0, 20.3)	18.5 (15.7, 21.5)	< 5
FE_{NO}	**21.3 (15.3, 27.4)***	15.5 (12.7, 18.3)	13.6 (11.0, 16.2)
Allergy (%)	9 (45%)	6 (32%)	11 (46%)

Data are presented as means (95% CI) unless otherwise stated. *Significantly different from non-athletes ($p<0.05$).

s, swimming; XC, cross-country skiing; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; FE_{NO}, fractional exhaled nitric oxide

Table 2 Differential cell counts in induced sputum (presented as proportion (differential %) and absolute numbers) and protein markers from athletes with and without asthma and healthy non-athletes given in medians (25^{th} to 75^{th} percentiles) unless otherwise stated.

	Asthmatic athletes			Healthy athletes			Non-athletes
	All (n=20)	Swimmers (n=10)	Cross-country skiers (n=10)	All (n=19)	Swimmers (n=10)	Cross-country skiers (n=9)	(n=24)
Total cells†/mg sputum	2217 (1036-5141)	4478 (1647-8863)	1733 (2028-2943)	2066 (981-2949)	2792 (1912-3345)	1241 (651-2258)	1790 (1454-2610)
Neutrophil granulocytes							
%*	38 (27-50)	34 (18-50)	42 (23-60)	36 (27-44)	38 (29-48)	31 (14-48)	31 (22-40)
cells/mg sputum	970 (244-1825)	1568 (317-2757)	735 (234-1297)	913 (240-1377)	1105 (719-1466)	360 (92-1194)	502 (302-772)
Airway macrophages							
%*	60 (50-72)	65 (49-81)	57 (38-75)	63 (55-72)	61 (51-70)	68 (51-85)	68 (58-77)
cells/mg sputum	1065 (668-3098)	2068 (1049-5693)	693 (648-1334)	1155 (670-1854)	168 (1056-2070)	699 (482-1786)	1333 (766-2028)
Lymphocytes							
%*	1.2 (0.6-1.8)	1.0 (0.4-1.6)	1.4 (0.4-2.5)	0.9 (0.6-1.2)	0.9 (0.5-1.4)	0.8 (0.4-1.2)	1.0 (0.7-1.3)
cells/mg sputum	22 (9-39)	31 (15-40)	15 (4-42)	13 (5-33)	19 (6-61)	6 (3-22)	12 (1-24)
Eosinophils							
%*	0.2 (0.0-0.4)	0.1 (0.0-0.2)	0.3 (0.0-0.7)	0.1 (0.0-0.2)	0.1 (0.0-0.3)	0.2 (0.0-0.3)	0.1 (0.0-0.3)
cells/mg sputum	0.0 (0.0-2.0)	0.0 (0.0-2.8)	0.0 (0.0-2.6)	0.0 (0.0-2.1)	0.0 (0.0-5.5)	0.0 (0.0-6.2)	0.0 (0.0-0.0)
Protein markers							
Sputum IL-8 (pg/ml)	378 (167-1123)	462 (169-1737)	356 (161-787)	340 (176-892)	863 (195-1127)	194 (168-446)	217 (130-314)
Sputum IL-1β (pg/ml)	9.6 (6.1-30.8)	10.2 (5.7-41.3)	8.9 (6.5-15.7)	12.6 (9.7-20.0)	13.1 (11.2-21.6)	11.6 (7.0-20.4)	9.0 (5.7-18.2)
Sputum CC16 (ng/ml)	2208 (642-4907)	2701 (635-6588)	2208 (959-3856)	2775 (871-3813)	3292 (1505-3974)	1837 (767-2847)	1332 (489-4043)
Plasma CC16 (ng/ml)	8.1 (6.3-9.6)	6.5 (3.3-8.1)	8.8 (7.8-10.4)	6.2 (5.3-8.3)	5.7 (4.3-9.4)	6.2 (5.4-7.7)	7.5 (6.5-8.8)

*Data presented as means (95% confidence intervals). †Leukocytes. CC16, Club Cell protein 16; IL, interleukin.

Table 3 Differential cell counts in induced sputum (presented as proportion) and protein markers from asthmatic and non-asthmatic swimmers (n=20) and cross-country skiers (n=19). Data are presented as means (95% confidence intervals) unless otherwise stated.

	PD_{20met} <2 µmol (n=9)	**PD_{20met} 2-4 µmol (n=5)**	**PD_{20met} >4-8 µmol (n=10)**	**PD_{20met} <8 µmol (n=15)**
Neutrophil granulocytes (%)	34 (20, 48)	25 (10, 41)	**49 (33, 64)**†	33 (21, 45)
Airway macrophages (%)	65 (50, 79)	72 (58, 89)	**51 (35, 66)**†	65 (53, 77)
Lymphocytes (%)	1.2 (0.5, 1.8)	1.2 (0.3, 2.0)	0.7 (0.0, 1.4)	1.1 (0.5, 1.8)
Eosinophils (%)	0.2 (0.0, 0.4)	0.1 (0.0, 0.3)	0.0 (0.0, 0.1)	0.2, (0.0, 0.5)
Protein markers				
Sputum IL-8 (pg/ml)*	354 (166, 1090)	437 (190, 580)	547 (187, 1227)	320 (170, 982)
Sputum IL-1β (pg/ml)*	9.0 (5.6, 27.7)	11.5 (5.6, 15.2)	11.7 (5.8, 29.3)	11.6 (7.6, 26.9)
Sputum CC16 (pg/ml)*	811 (559, 6710)	1996 (1851, 2825)	2847 (1611, 4472)	2738 (809, 3473)
Plasma CC16 (pg/ml)*	5.8 (3.6, 7.7)	8.8 (6.8, 9.4)	7.0 (6.2, 9.2)	8.2 (5.6, 9.2)

*Data presented in medians (25^{th} to 75^{th} percentiles). †Significantly different from PD_{20met} 2-4 µmol ($p<0.05$).
PD_{20met}, The inhaled dose of methacholine causing a 20% decrease in the forced expiratory volume in one second (FEV_1); CC16, Club Cell protein 16; IL, interleukin.

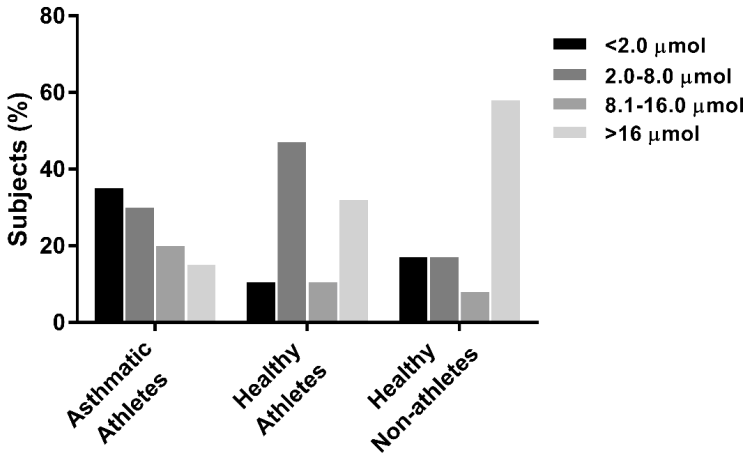

Figure 1 Severity of bronchial hyperresponsiveness (BHR) defined as the methacholine dose (μmol) causing ≥20% decrease in forced expiratory volume in one second (FEV_1) (PD_{20met}) in 20 athletes with asthma, 19 healthy athletes and in 24 healthy non-athletes. The distribution in PD_{20met} differed among groups (p=0.005).

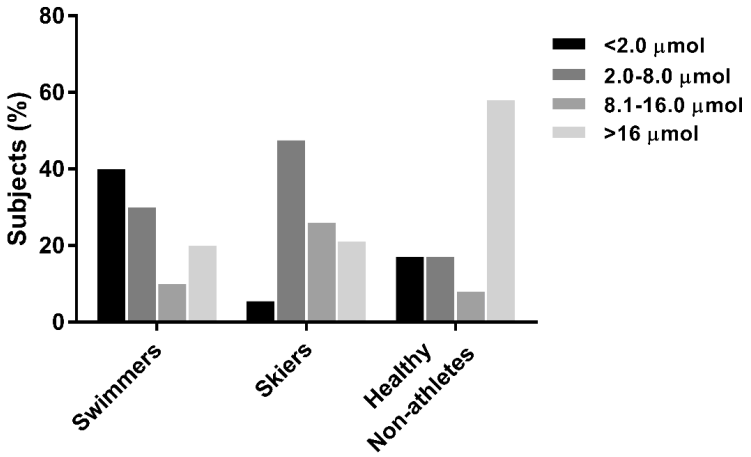

Figure 2 Severity of bronchial hyperresponsiveness (BHR) defined as the methacholine dose (μmol) causing ≥20% decrease in forced expiratory volume in one second (FEV_1) (PD_{20met}) in 20 swimmers, 19 cross-country skiers, and 24 healthy non-athletes. The distribution in PD_{20met} differed among groups (p=0.007).

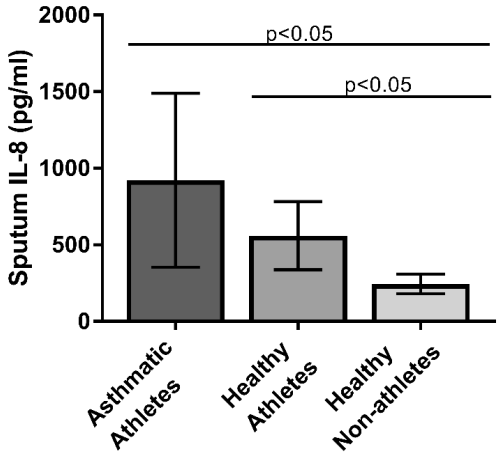

Figure 3 Sputum interleukin (IL)-8 in three groups; Athletes with asthma (n=20), healthy athletes (n=19) and healthy non-athletes (n=24) presented as median with interquartile range. Error bars represent maximal and minimal values. P-values show difference between healthy non-athletes and the other groups.

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ORIGINAL ARTICLE

Two distinct phenotypes of asthma in elite athletes identified by latent class analysis

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Abstract

Introduction: Clusters of asthma in athletes have been insufficiently studied. Therefore, the present study aimed to characterize asthma phenotypes in elite athletes using latent class analysis (LCA) and to evaluate its association with the type of sport practiced. *Methods*: In the present cross-sectional study, an analysis of athletes' records was carried out in databases of the Portuguese National Anti-Doping Committee and the Norwegian School of Sport Sciences. Athletes with asthma, diagnosed according to criteria given by the International Olympic Committee, were included for LCA. Sports practiced were categorized into *water*, *winter* and *other* sports. *Results*: Of 324 files screened, 150 files belonged to asthmatic athletes (91 Portuguese; 59 Norwegian). LCA retrieved two clusters: "*atopic asthma*" defined by allergic sensitization, rhinitis and allergic co-morbidities and increased exhaled nitric oxide levels; and "*sports asthma*", defined by exercise-induced respiratory symptoms and airway hyperresponsiveness without allergic features. The risk of developing the phenotype "*sports asthma*" was significantly increased in athletes practicing water (OR = 2.87; 95%CI [1.82–4.51]) and winter (OR = 8.65; 95%CI [2.67–28.03]) sports, when compared with other athletes. *Conclusion*: Two asthma phenotypes were identified in elite athletes: "*atopic asthma*" and "*sports asthma*". The type of sport practiced was associated with different phenotypes: water and winter sport athletes had three- and ninefold increased risk of "*sports asthma*". Recognizing different phenotypes is clinically relevant as it would lead to distinct targeted treatments.

Keywords

Asthma, athletes, clusters, exercise-induced bronchoconstriction, latent class analysis, phenotypes, sports, training environment

History

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Introduction

Exercise training improves asthma symptoms, quality of life, exercise capacity, bronchial hyperesponsiveness (BHR) and lung function in asthmatics [1,2]. Thus, physical activity should be recommended as a supplementary therapy to medication in asthmatic subjects [1]. However, although moderate exercise has proven to be beneficial, repeated high-intensity exercise performed by elite athletes seems to contribute to the development of asthma and BHR. In fact, it has been recognized that elite athletes have increased risk of developing asthma, especially those who practice endurance sports, such as swimming and running, or winter sports [3,4]. Nevertheless, asthma is a complex syndrome with variable clinical presentation, and different physiologic and pathologic parameters. Characterization of this heterogeneity has promoted the concept of asthma consisting in multiple phenotypes or consistent groupings of characteristics [5].

Defining phenotypes of asthma has been a major objective in recent years, as it would facilitate research into etiology and pathophysiology, targeted treatment and preventive measures, and improve prediction of long-term outcomes [6]. Up to this moment, in what concerns athletes with asthma, there is no evidence to support clusters of grouping characteristics, although it is generally recognized that the asthmatic condition which develops in athletes during their sports career is not likely to be similar to what is usually considered to be asthma in clinical practice [7]. The hypothesis of different phenotypes of asthma occurring in athletes has only been approached once in the literature, in a review article. Haahtela et al. [8] suggested that there may be two different clinical phenotypes of asthma in elite athletes: ''classical asthma'' characterized by early onset childhood asthma, methacholine hyperesponsiveness, atopy and signs of

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eosinophilic airway inflammation reflected by increased exhaled nitric oxide levels (FE_{NO}); and another distinct phenotype with late onset of symptoms during sports career, airway responsiveness to eucapnic voluntary hyperpnoea (EVH) and a variable association with atopic markers and FE_{NO}. These phenotypes were described only in Finnish athletes, and have not been fully established so far.

Most recent efforts to describe phenotypes are based on cluster analysis. These multivariate statistical methods allow splitting the differences between patient group data into disease categories and clinically meaningful groups, therefore being less dependent on *a priori* assumptions. These methods have already been successfully applied within respiratory medicine [6,9–11] to identify asthma phenotypes that exhibited differences in clinical, physiological and inflammatory parameters as well as response to treatment [10,11]. However, such methods have not been applied to athletes with asthma.

The objectives of the present study were to identify and characterize asthma phenotypes in elite athletes using latent class analysis (LCA) and to assess a possible association with the type of sport practiced.

Methods

Design and participants

In the present cross-sectional study, an analysis of elite athlete records kept in database files of two different countries was performed. Portuguese and Norwegian athletes training at high competitive levels (national, international or Olympic teams) were identified through existing institution databases. In Portugal, we used registries of elite athletes available at the Portuguese Anti-doping Authority and the Portuguese database of Olympic athletes; in Norway, we analyzed medical files from the respiratory medical team of the Norwegian School of Sport Sciences, including Olympic athletes participating in the 2008 summer and 2010 winter Olympic Games. Athletes were selected according with available information on symptoms, lung function and airway inflammation, BHR, and allergic sensitization. Healthy athletes and those with other conditions rather than asthma were excluded. From all reviewed files, 324 files had complete information available and informed consent for data use. Of these 324 athletes, 150 athletes fulfilled asthma criteria and were included for LCA.

The present study was conducted in accordance with Declaration of Helsinki for Medical Research Involving Human Subjects and was approved by Regional Medical Ethics Committees and Norwegian Data Inspectorate. All included subjects signed an informed consent for data usage.

Definitions

Asthma diagnosis was established by a medical doctor according to criteria set by the International Olympic Committee to document asthma in athletes [4,12], with objective evidence of either reversibility after bronchodilator administration or BHR after a bronchial provocation challenge. The demographic data obtained included age, gender, height, weight and sport practiced. The type of sport was classified according to environmental training conditions into *water* sports (swimming and water polo), *winter* sports (cross-country skiing, biathlon, skeleton, alpine skiing and ski cross) and *other* sports (speed skating, curling, handball, judo, triathlon, football, cycling, beach volley, rowing, athletics, sailing, badminton, canoeing, curling, equestrian, taekwondo, auto-racing, billiards, paragliding, rugby, tennis, roller hockey, kickboxing, fencing, basketball or golf). Medical data collected included presence of respiratory symptoms, current use of asthma medication and presence of rhinitis or other allergic diseases (conjunctivitis, urticaria, eczema, anaphylaxis and drug, food and venom allergies). These data were sampled through allergy questionnaire for athletes (AQUA) questionnaire [13] at the time of the medical consultation. For statistical purposes, variables were categorized according to the definitions presented in Table 1. Spirometry was performed in agreement with the European Respiratory Society guidelines [14] and results (forced expiratory volume in first second – FEV_1 and forced vital capacity – FVC) were presented as both absolute and predicted values, according to published reference algorithms [15]. For both airflow obstruction and BHR, the first ever performed spirometry and the first ever performed bronchial provocation challenge, respectively, were considered.

Statistical analysis

Results are presented as mean values [95% confidence interval (CI)], mean ± standard deviation (SD), or medians ± interquartile range (IQR) in case of skewed distribution, or counts (*n*, %). Independent samples *t*-test was used for comparison of normally distributed continuous data, and Mann–Whitney test was used on data with skewed distribution. Categorical variables were compared by Chi-square or Fisher's exact tests. These analyses were performed using SPSS (IBM SPSS Statistics for Windows, Version 20.0, IBM Corp., Armonk, NY), considering a significance level of 0.05.

LCA was used to uncover distinct groups of individuals from a sample (patterns) homogeneous within the group, considering that the performance of an individual in a set of items is explained by a categorical latent variable with K classes, commonly called ''latent classes''. Model interpretation was based on item profiles in each category and obtained from probabilities of endorsing each item response, conditional on class membership. In the present study, the number of latent classes was defined according to Bayesian Information Criterion (BIC). Starting from one single class and increasing one class at each step, the best solution was identified when the increase of number of classes did not lead to a decrease in BIC. LCA used nine variables important for asthma definition or relevant for differential diagnosis (Table 1). The selection of variables was based on the assumption of their clinical relevance for asthma definition. The Lo–Mendell–Rubin likelihood ratio test of model fit was used to quantify the likelihood that the data could be described by a model with one-less class. All LCA models were fitted using MPlus (V.5.2; Muthen & Muthen, Los Angeles, CA). Later, among asthmatic athletes, we estimated the risk associated with the sport training environment, by using regression

Table 1. Definitions of variables set for LCA.

Variable	Definition
Airflow obstruction	FEV_1/FVC ratio lower than 0.70
Reversibility	Increase of at least 200 mL and 12% in FEV_1
Rhinitis[a]	Positive answer to the question ''Did any doctor diagnose you an allergic disease?'' AND ''Rhinitis'' OR Positive answer to the question ''Do you frequently sneeze, have a running, itchy nose (apart from colds)?
Any other allergic disease[a]	Positive answer to the question ''Did any doctor diagnose you an allergic disease?'' (except rhinitis) OR Positive answer to the question ''Have you frequently red eyes with tearing and itching?'' OR Positive answer to the question ''Have you ever had severe allergic or anaphylactic reactions?'' OR Positive answer to the question ''Have you ever had allergic reactions to foods?'' OR Positive answer to the question ''Have you ever had allergic reactions to drugs?''
Respiratory symptoms[a]	Self-reported recurrent breathlessness, cough, wheezing, chest tightness and/or phlegm production OR Positive answer to the question ''Did any doctor diagnose you an allergic disease?'' AND ''Asthma'' OR Positive answer to the question ''Have you ever had shortness of breath, cough and/or itching of the throat following exercise?''
Asthma treatment	Current or recent treatment with ICS and/or $Beta_2$-agonists
Airway hyperesponsiveness[b]	A fall in FEV_1 $\geq$10% from baseline with exercise or EVH OR A fall in FEV_1 $\geq$15% from baseline after inhaling 22.5 ml of 4.5 g% NaCl or $\leq$635 mg of mannitol OR A fall in FEV_1 $\geq$20% from baseline with methacholine: PC_{20} $\leq$4 mg/ml, or PD_{20} $\leq$400 µg (cumulative dose) or $\leq$200 µg (noncumulative dose) in those not taking ICS, and PC_{20} $\leq$16 mg/ml or PD_{20} $\leq$1600 µg (cumulative dose) or $\leq$800 µg (noncumulative dose) in those taking ICS for at least 1 month
Eosinophilic inflammation	The presence of FE_{NO} levels above 25 ppb
Allergic sensitization	The presence of at least one positive (mean of largest and perpendicular diameter of the wheal $\geq$3 mm for each allergen and controls showing adequate reactions) skin prick test or the presence of positive specific IgE ($\geq$0.35 kU/L) for at least one common aeroallergen in the local geographic area

EVH, eucapnic voluntary hyperpnoea; FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; ICS, inhaled corticosteroids; FE_{NO}, exhaled nitric oxide; PD_{20}, provocative dose of methacholine causing a 20% decrease in FEV_1; PC_{20}, provocative concentration of methacholine causing a 20% decrease in FEV_1.
[a]Considering the AQUA questionnaire.
[b]According to International Olympic Committee Medical Commission to diagnose asthma in athletes.

analysis to predict the odds of having a specific asthma pattern (phenotype), having ''other sports'' as reference.

Results

Included subjects

From 324 files reviewed, 150 files belonged to athletes who fulfilled asthma criteria (91 Portuguese; 59 Norwegian). Forty-five athletes were diagnosed with asthma based on positive bronchodilation (the mean ± SD of FEV_1 increase was 450 mL ± 292 and 13% ± 9.4), and 105 by presenting airway responsiveness after a provocation challenge: 1 positive challenge to mannitol, 3 positive challenges with exercise and the remaining 101 positive challenges with methacholine (7 reporting PC_{20}: mean 3.9 mg/mL; 94 reporting PD_{20}: mean 6.8 µg). The remaining athletes were healthy ($n = 129$) or had other pathologic conditions ($n = 45$). Asthmatic subjects included in the present study presented airflow limitation, more reversibility to salbutamol, more BHR, atopy, rhinitis and airway inflammation assessed by FE_{NO} (Table 2).

LCA model

Relying on asthma defining variables, the increase in likelihood values leveled off when increasing from one to two classes, and BIC reached its optimum value at two classes (Online Table). This result was confirmed by Lo–Mendell–Rubin likelihood ratio test.

Class 1 was characterized by allergic sensitization, rhinitis and other allergic co-morbidities, and increased FE_{NO} levels (''*Atopic asthma*''); while class 2 was characterized by the occurrence of respiratory symptoms and BHR, in the absence of atopic features (''*Sports asthma*'') (Table 3 and Figure 1).

Subject's differences between classes

The athletes which were assigned to ''atopic asthma'' presented higher values of FE_{NO} than those in ''sports asthma'' (32.2 vs. 15.7, $p = 0.002$). In ''atopic asthma'', 28 athletes presented increased values of FE_{NO}, compared to only 7 among those in ''sports asthma''.

Allergic diseases were evident in 60.7% of athletes in ''atopic asthma'', and in 12.5% of those assigned to ''sports asthma'', namely: conjunctivitis (48% of athletes in ''atopic asthma'' and none in ''sports asthma''), atopic eczema (12% of athletes in ''atopic asthma'' and none in ''sports asthma''), and food allergy (31% of athletes in ''atopic asthma'' and none in ''sports asthma''). Hymenoptera venom allergy, drug allergy and anaphylaxis had a similar prevalence in both

Table 2. Features of athletes screened at Portuguese National Anti-Doping Organization and at Norwegian School of Sports Sciences databases.

	Asthmatic athletes ($n = 150$)	Non-asthmatic athletes ($n = 174$)	p
Male, *n* (%)	107 (71)	89 (51)	**<0.001**[e]
Age, years	25 (14–40)	26 (16 – 38)	0.251[d]
BMI, kg/m^2	23 [23;24]	23 [22;23]	0.06[c]
Physician reported rhinitis, *n* (%)	54 (36)	33 (19)	**0.003**[e]
Other allergic disease, *n* (%)	20 (13)	26 (15)	0.750[e]
Atopy, *n* (%)	89 (59)	58 (33)	**<0.001**[e]
Respiratory symptoms, *n* (%)*	138 (92)	89 (51)	**<0.001**[e]
Dyspnea/heavy breathing	48 (32)	20 (11)	**<0.001**[e]
Chest tightness	12 (8)	11 (6)	0.379[e]
Wheezing	42 (28)	15 (9)	**<0.001**[e]
Cough	44 (29)	33 (19)	**0.002**[e]
Tiredness	1 (0.7)	1 (0.6)	0.427[f]
Phlegm	18 (12)	15 (9)	**<0.001**[e]
Asthma treatment, *n* (%)			**<0.001**[f]
Inhaled steroids alone	9 (6)	1 (0.6)	
Beta-2-agonists alone	13 (9)	2 (1)	
Inhaled steroids + β2-agonists	96 (64)	13 (8)	
Airway obstruction[a], *n* (%)	43 (29)	21 (12)	**<0.001**[e]
FVC			
L	5.4 [5.1;5.7]	5.2 [5.0;5.4]	0.41[c]
% of predicted	114 [110;117]	112 [109;116]	0.60[c]
FEV_1			
L	4.1 [3.9;4.4]	4.3 [4.1;4.4]	0.06[c]
% of predicted	101 [96;106]	109 [106;111]	**0.001**[c]
FEV_1/FVC	69 [65;74]	76 [72;80]	**0.012**[c]
Reversibility[b], *n* (%)	26 (17)	1 (0.6)	**0.037**[f]
Airway hyperesponsiveness, *n* (%)	126 (84)	51 (29)	**<0.001**[e]
FE_{NO}, ppb	33 (6–213)	19 (4–70)	**0.01**[d]

Bold values indicate $p < 0.05$.
Data presented as mean (95% confidence interval) except for age and FE_{NO} which are presented as median (min–max).
BMI, body mass index; FE_{NO}, exhaled fraction of nitric oxide; L, liters; FVC, forced vital capacity; FEV_1, forced expiratory volume in one second.
[a]Defined as a FEV_1/FVC ratio <0.70.
[b]Defined as an increase in FEV_1 ≥200 mL and ≥12%.
[c]Independent samples *t*-test.
[d]Independent samples Mann–Whitney *U* test.
[e]Chi-square test.
[f]Fisher's exact test.

classes (4% of athletes for both diseases). Male gender was predominant in ''sports asthma''.

Regarding therapeutic, 92.5% of those athletes with ''atopic asthma'' and 78% of those with ''sports asthma'' were under anti-asthmatic drugs. Thirteen asthmatic athletes were using only short-acting β2-agonists as therapeutic – 9 (8%) among the ''atopic asthma'' and 4 (8%) among the ''sports asthma'' phenotype; the remaining athletes were on inhaled corticosteroids (ICS) alone or combined with long-acting β2-agonists.

Risk factors for each class

A 2.87 (95%CI: 1.82–4.51) and 8.65 (95%CI: 2.67–28.03) fold increase for risk of ''*sports asthma*'' was observed in athletes practicing water sports and winter sports, respectively, when compared to other sports (Figure 2).

Discussion

Using LCA, this present study identifies two distinct phenotypes of asthma in athletes: ''*atopic asthma*'' defined by the occurrence of atopy, increased levels of FE_{NO}, rhinitis and other allergic co-morbidities; and ''*sports asthma*'', defined by the presence of exercise-induced respiratory symptoms and BHR in the absence of allergic features. Moreover, specific training and environmental conditions are associated with an increased risk of developing ''*sports asthma*'', as athletes practicing water and winter sports had, respectively, a three- and ninefold increase in their risk of ''*sports asthma*'', when compared with others.

This study allows for hypothesis generation and has several strengths. Its major strength is the new type of statistical models used to pool and characterize different clusters. This methodological approach makes this study especially useful by retrieving a clear view on asthma phenotypes in athletes. Replication of results in other datasets is important when using these exploratory statistical techniques; and the two asthma patterns obtained in this study are remarkably in accordance with the only previous report, a study relying on different study design and an *a priori* list of selected variables for statistical analysis [8]. Another strength of the present study is its multicentric nature, allowing the inclusion of a large sample of elite athletes, all competing at top levels, some of which are among the world's best in their discipline with several winners of Olympic Gold medals.

Athletes in this study are all competing in an elite level and, therefore, all are more prone to negative consequences of exercise ''injuring'' airways due to prolonged and repeated

Table 3. Characteristics of asthmatic athletes according with their asthma phenotype and variables in each assigned latent class.

	Total	Atopic asthma, $n = 104$	Sports asthma, $n = 46$	p
Male, *n* (%)	107	81 (78)	26 (57)	**0.008**[e]
Age, median ± IQR in years	–	23.0 ± 12	24.5 ± 8	0.522[f]
Height, mean ± SD in cm	–	175.4 ± 8.7	176.5 ± 8.7	0.530[g]
Weight, mean ± SD in kg	–	70.9 ± 11.5	71.4 ± 10.3	0.815[g]
BMI, mean ± SD in kg/m^2	–	23.0 ± 2.6	22.8 ± 1.9	0.741[g]
FEV_1, mean ± SD in L	–	4.0 ± 0.9	4.1 ± 0.7	0.221[g]
FEV_1, mean ± SD in % predicted	–	98.1 ± 20.4	99.7 ± 21.1	0.640[g]
FVC, mean ± SD in L	–	5.1 ± 1.0	5.3 ± 1.1	0.413[g]
FVC, mean ± SD in % predicted	–	108.0 ± 15.4	113.4 ± 15.0	0.084[g]
FEV_1/FVC, mean ± SD	–	77.9 ± 8.9	78.7 ± 11.1	0.649[g]
Variables used in LCA				
Airflow obstruction[a]				**0.036**
No	80.5	85.3	69.4	
Yes	19.5	14.7	30.6	
Reversibility[b]				**0.023**
No	23.4	19.0	39.7	
Yes	76.6	81.0	60.3	
Rhinitis				**< 0.001**
No	64.0	51.5	90.9	
Yes	36.0	**48.5**	9.1	
Any other allergic disease[c]				**<0.001**
No	61.5	39.3	87.5	
Yes	38.5	**60.7**	12.5	
Respiratory symptoms				0.133
No	6.1	4.0	10.7	
Yes	93.9	96.0	89.3	
Asthma treatment				**0.017**
No	11.9	7.5	22.0	
Yes	88.1	92.5	78.0	
Airway hyperesponsiveness				0.834
No	25.7	25.0	26.9	
Yes	74.3	75.0	73.1	
FE_{NO}[d]				**<0.001**
Normal	62.8	44.8	84.5	
Increased	37.2	**55.2**	15.5	
Atopy				**<0.001**
No	31.0	0	100	
Yes	69.0	**100**	0	

Bold values indicate $p < 0.05$.

Data presented as percentage of total, except otherwise stated. BMI, body mass index; FE_{NO}, exhaled fraction of nitric oxide; L, liters; FVC, forced vital capacity; FEV_1, forced expiratory volume in one second.

[a]Defined as a FEV_1/FVC ratio <0.70.

[b]Defined as an increase in FEV1 ≥200 mL and ≥12%.

[c]Other allergic diseases include conjunctivitis, urticaria, eczema, anaphylaxis and drug, food and venom allergies, sampled through AQUA questionnaire.

[d]Defined as increased if above 25 ppb.

+Chi-square test.

[f]Mann–Whitney *U* test.

hyperpnoea. For athletes practicing water and winter sports, in addition to frequent episodes of prolonged hyperpnoea, their ''occupation'' demands exposure to potentially noxious stimuli, such as sport-specific environmental exposures [16]. Keeping in mind the close relation to environmental conditions, one could speculate whether ''*sports asthma*'' should be classified as a variant of occupational asthma, as recently suggested [16]. This designation could help improve the general idea of this concept of asthma dependent upon environmental factors which are part of an athlete's occupation. The ''sports asthma'' phenotype is similar to the late-onset phenotype identified among ''normal'' asthmatics. In many cases, the late-onset phenotype appears to be more severe, less responsive to standard therapy and more related to environmental risk factors [17]. However, ''sports asthma'' tends to improve after cessation of sport participation, in what concerns airway inflammation and hyperesponsiveness [18,19].

In athletes, atopy has been long recognized to be positively associated with asthma and BHR [20,21]. Moreover, training in cold air [21] and swimming [20] were identified as risk factors for asthma. In both swimmers and cross-country skiers, the prevalence of asthma is known to increase with age [22–24], which is consistent with the hypothesis of ''sports asthma'' occurring throughout the sport career and being induced by cumulative years of exposure to environmental training conditions. The results of our study contribute to confirm that different risk factors, such as atopy and environmental training conditions, result in different patterns of asthma. The effect of these risk factors on determining different underlying mechanisms of asthma should be considered.

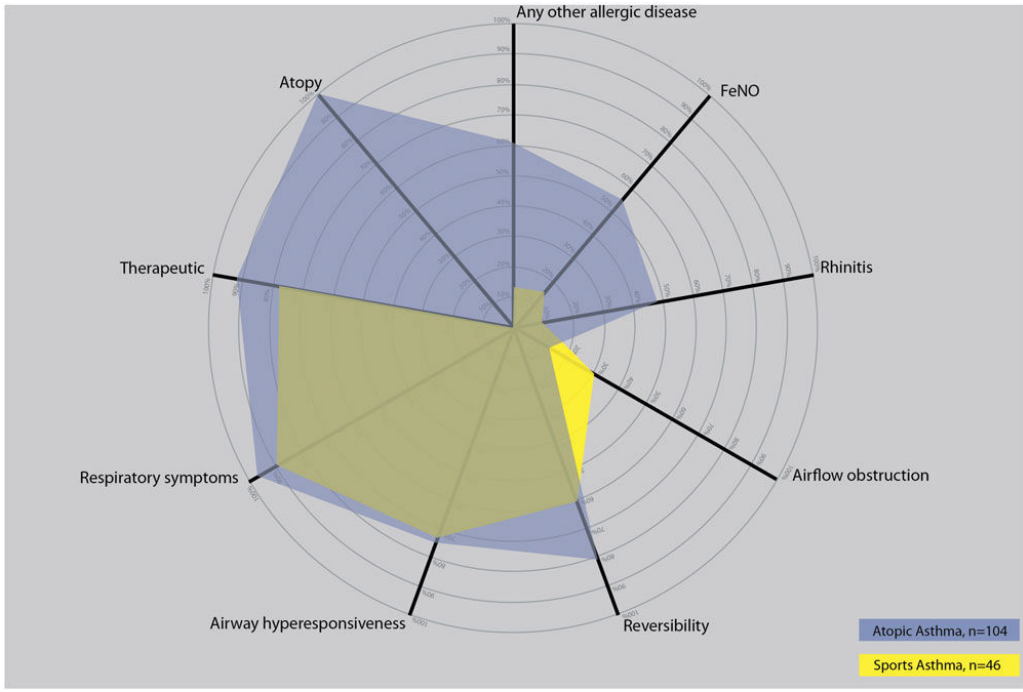

Figure 1. Percent of athletes presenting each of the variables included for LCA.

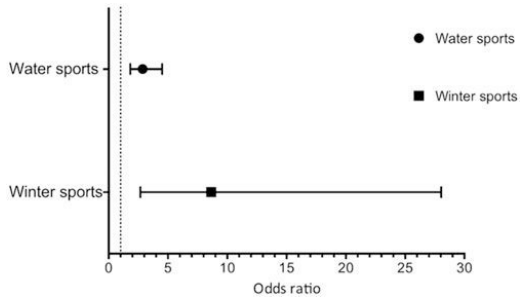

Figure 2. Risk of presenting the ''sports asthma'' phenotype of athletes practicing water and winter sports, considering other sports as reference.

Defining these distinct phenotypes could lead not only to further understanding the underlying mechanisms of asthma in elite athletes, but also, and most important from a practical point of view, to recognizing that potentially different treatments specifically targeted for defined phenotypic groups are needed. Optimal asthma treatment is a prerequisite for asthmatic athletes because of potential implications in performance, since airway narrowing during exercise could compromise ventilatory capacity and efficiency. However, it has been recognized that the natural course of asthma in athletes is difficult to change by ''normal'' anti-inflammatory treatment [25]. This highlights the need for a different therapeutic approach in these subjects, which leads us to *the clinical implications of our study.* Differences in airway response to bronchodilating drugs have been reported in the literature, and whether athletes with asthma occurring during sports career respond to anti-asthmatic drugs similarly to subjects with classic allergic or with nonallergic asthma has not been extensively studied [7] and needs further research. Most recent guidelines for treatment of exercise-induced bronchoconstriction (EIB) state a strong recommendation for using a short-acting β2-agonist before exercise in *all* patients with EIB [26]. However, we have recently shown that elite skiers with asthma respond better to anticholinergic treatment as compared with β2-agonists [27]. Differences in parasympathetic bronchial tone were suggested as a possible explanation to why some subjects are responders and other non-responders to anticholinergic drugs [28,29]. It seems, therefore, that the approach of ''*one treatment fits all*'' *is insufficient* to comply with the needs of asthmatic athletes.

Despite its several strengths, our study also has some limitations that must be pointed out. The first is the use of different methods (both direct and indirect challenges) to assess BHR in athletes. In the present study, information was collected from medical files, so there was no possibility to homogenize tests performed by athletes in two centers. In any case, final diagnosis was made according to IOC criteria. Another weakness to be noted is the absence of information about age of asthma onset; this limits the extent of our conclusions as we cannot be aware of whether the previous presence of asthma would influence the type of sport chosen. However, based on previous literature, it does not seem to be

the case as the prevalence of asthma is known to increase with age both in swimmers and skiers [22–24]. The interpretation of our results is also limited by the cross-sectional design, which is not able to identify causality; however, it is suitable for hypothesis generation. Thus, the present study should be succeeded by new prospective studies following youth athletes from adolescence until adulthood. Moreover, although motivating, results provided by this exploratory analysis have to be interpreted in context of future work, addressing whether the two phenotypes are relevant from a clinical perspective. Potential phenotypes require prospective validation with clinical interventional trials. A recent trial showed that Norwegian competitive endurance winter athletes respond with a higher reversibility to ipratropium bromide than to inhaled β2-agonists [27], helping research in this field to move forward and toward a new direction.

Conclusion

Using LCA on a large sample of top elite athletes from two national databases we were able to identify two patterns of asthma aggregation features based on findings routinely collected in clinical practice: ''*atopic asthma*'', defined by the presence of allergic sensitization, rhinitis and other allergic co-morbidities and increased FE_{NO}; and ''*sports asthma*'', defined by the presence of exercise-induced respiratory symptoms and BHR in the absence of allergic features. Moreover, exposure to particular environmental conditions of training and competition was associated with increased risk to develop ''*sports asthma*'' phenotype: water sports increased the risk by almost three times, whereas in winter sports the risk increased by almost nine times. Recognizing different phenotypes as a result of probable different underlying mechanisms related to environmental exposures highlights the need for distinct targeted treatments. These potential phenotypes require prospective validation by larger clinical interventional trials. If confirmed by other studies, such a model could be useful for the standardization of clinical diagnosis and future treatment of asthmatic athletes.

Acknowledgements

We thank to Hugo Martins, for his contribution in image designing of Figure 1.

Declaration of interest

The authors report no conflicts of interest. To European Academy of Allergy and Clinical Immunology for the 2011 Exchange Research Fellowship award allowing the first author to work in Oslo and therefore turned this project possible.

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Supplementary material available online

Appendix I

Approval letters from the Regional Committees for Medical Research Ethics

- **study I (2013/167)**
- **study II (S-07468a & 174/12)**

REK
REGIONALE KOMITEER FOR MEDISINSK OG HELSEFAGLIG FORSKNINGSETIKK

Region:	Saksbehandler:	Telefon:	Vår dato:	Vår referanse:
REK sør-øst	Tor Even Svanes	22845521	07.03.2013	2013/167/REK sør-øst C
			Deres dato:	**Deres referanse:**
			22.01.2013	

Vår referanse må oppgis ved alle henvendelser

Kai-Håkon Carlsen
Oslo Universitetssykehus

2013/167 Er toppidrett skadelig for luftvegene?

Forskningsansvarlig: Oslo Universitetssykehus
Prosjektleder: Kai-Håkon Carlsen

Vi viser til søknad om forhåndsgodkjenning av ovennevnte forskningsprosjekt. Søknaden ble behandlet av Regional komité for medisinsk og helsefaglig forskningsetikk (REK sør-øst) i møtet 14.02.2013. Vurderingen er gjort med hjemmel i helseforskningsloven (hfl.) § 10, jf. forskningsetikklovens § 4.

Prosjektomtale
Forekomst av astma og bronkial hyperreaktivitet (BHR) er svært høy i kondisjonsidrett, særlig vinteridrett (langrenn, skiskyting) og svømming (>50% på landslagsnivå). Årsaken er ukjent Hensikten med studien er å klarlegge mekanismer for bedret forståelse som kan forebygge astma. Det vil være en case-kontroll studie der 30 toppidrettsutøvere med astma, 30 uten astma og 30 friske kontroller, i alderen 16-40 år, skal inkluderes. Man vil registrere lungefunksjon, BHR (metakolinprovokasjon)luftvegs-inflammasjon og -epitelskade (indusert sputum, ekshalert pustekondensat), prikktest, parasympatisk aktivitet (pupillometri og variasjon i cardialaktivitet)spyttkortisol, xenobioticaeksponering. Deretter skal man analysere sammenheng mellom faktorer og utvikling av astma og BHR. Studien er samtykkebasert, og det vil opprettes en spesifikk forskningsbiobank.

Vurdering
Komiteen har ingen innvendinger til designet i studien.

Forskningsbiobank
Det søkes om å opprette en spesifikk forskningsbiobank med navn Er toppidrett skadelig for luftvegene? i prosjektet.

Ansvarshavende for forskningsbiobanken er Wenche Reed. Forskningsansvarlig er Oslo Universitetssykehus.

Biobanken vil bestå av blodprøver, urinprøver, spyttprøver, indusert sputum og luftveiskondensat.

Biobanken planlegges å vare til 2028. Deretter skal materialet behandles i henhold til helseforskningslovens § 30.

Biologisk materiale vil potensielt utføres til utlandet i henhold til helseforskningslovens § 37. Deltakerne er orientert om dette i informasjonsskriv.

Informasjonsskriv og samtykkeerklæring
Informasjonsskrivet er sterkt preget av fagterminologi og medisinske begreper. Skrivet er dessuten langt. Begge deler gjør informasjonen til deltakerne mindre tilgjengelig enn den hadde trengt å være. Det bes om at prosjektleder gjennomgår skrivet med tanke på å gjøre det mer allmenngyldig.

Det bes videre om at selve samtykkeerklæringen flyttes til etter kapittel A og B av skrivet. Samtykkeerklæringen skal komme etter at all relevant informasjon er gitt.

Endelig bes det om at det anføres at REK sør-øst har godkjent studien. I det foreliggende skrivet står det at REK har vurdert studien og ikke har innvendinger.

Ut fra dette setter komiteen følgende vilkår for prosjektet:

1. Informasjonsskriv skal revideres i tråd med det ovennevnte, og sendes komiteen til orientering.

Vedtak
Prosjektet godkjennes under forutsetning av at ovennevnte vilkår oppfylles, jf. helseforskningslovens §§ 9 og 33.

I tillegg til vilkår som fremgår av dette vedtaket, er tillatelsen gitt under forutsetning av at prosjektet gjennomføres slik det er beskrevet i søknaden og protokollen, og de bestemmelser som følger av helseforskningsloven med forskrifter.

Tillatelsen gjelder til 31.12.2018. Av dokumentasjons- og oppfølgingshensyn skal opplysningene likevel bevares inntil 31.12.2023. Opplysningene skal lagres avidentifisert, dvs. atskilt i en nøkkel- og en opplysningsfil. Opplysningene skal deretter slettes eller anonymiseres, senest innen et halvt år fra denne dato.

Komiteens avgjørelse var enstemmig.

Sluttmelding og søknad om prosjektendring
Prosjektleder skal sende sluttmelding til REK sør-øst på eget skjema senest 15.08.2016, jf. hfl. 12. Prosjektleder skal sende søknad om prosjektendring til REK sør-øst dersom det skal gjøres vesentlige endringer i forhold til de opplysninger som er gitt i søknaden, jf. hfl. § 11.

Klageadgang
Du kan klage på komiteens vedtak, jf. forvaltningslovens § 28 flg. Klagen sendes til REK sør-øst. Klagefristen er tre uker fra du mottar dette brevet. Dersom vedtaket opprettholdes av REK sør-øst, sendes klagen videre til Den nasjonale forskningsetiske komité for medisin og helsefag for endelig vurdering.

Forskningsprosjektets data skal oppbevares forsvarlig, se personopplysningsforskriften kapittel 2, og Helsedirektoratets veileder for Personvern og informasjonssikkerhet i forskningsprosjekter innenfor helse og omsorgssektoren.

Vi ber om at alle henvendelser sendes inn med korrekt skjema via vår saksportal: http://helseforskning.etikkom.no. Dersom det ikke finnes passende skjema kan henvendelsen rettes på e-post til: post@helseforskning.etikkom.no.

Med vennlig hilsen

Arvid Heiberg
prof. dr.med
leder REK sør-øst C

Tor Even Svanes
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Telefaks: 228 44 661
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Nettadresse: www.etikkom.no

Dato: 20.12.07
Deres ref.:
Vår ref.: S-07468a

S-07468a Astma og allergi hos Olympiere [1.2007.2840]

Vi viser til søknad mottatt 07.11.07 med følgende vedlegg: Protokoll; foreløpig spørreskjema; informasjonsskriv med samtykkeerklæring; søknad om forskningsbiobank (gammelt skjema); søknad om forskningsbiobank melding nr. 2145, datert 9. november 2007.

Komiteen behandlet søknaden i sitt møte onsdag 12. desember 2007. Prosjektet er vurdert etter lov om behandling av etikk og redelighet i forskning av 30. juni 2006, jfr. Kunnskapsdepartementets forskrift av 8. juni 2007 og retningslinjer av 27. juni 2007 for de regionale komiteer for medisinsk og helsefaglig forskningsetikk.

Prosjektet er en internasjonal studie som skal undersøke olympiske deltakere med tanke på forekomst og medisinske behov på grunn av astma og allergi. I tillegg er det et mål å utvikle et optimalt studieverktøy for å undersøke slike sykdommer hos idrettsutøvere. Det tas sikte på å rekruttere i alt 2000 deltakere i olympiske leker. I Norge er målet å rekruttere 200 idrettsutøvere. I første omgang rekrutteres deltakere i førstkommende sommerolympiade. Studien er inndelt i flere faser, og norske deltakere forutsettes å delta i alle faser.

Etiske problemstillinger knyttet til de medisinske undersøkelser av deltakerne, blant annet metakolin-test på friske personer, er drøftet i søknaden.

Komiteen har følgende merknad til informasjonsskriv/samtykkeerklæring:

> Det må gjerne innhentes bekreftelse fra den som informerer deltakeren om at informasjon er gitt; men signaturen skal ikke sidestilles med prosjektdeltakers signatur i samtykkeerklæringen og dermed fremstå som en medundertegning. En evt. underskrift av den som innhenter samtykket, skal tydelig fremstå som en bekreftelse på at informasjon er gitt.

Vedtak:
Prosjektet godkjennes under forutsetning av at den merknaden som er anført ovenfor, blir innarbeidet før prosjektet settes i gang.

Komiteen videresender skjema for opprettelse av forskningsbiobank og informasjonsskrivet samt komiteens vedtak til Sosial- og helsedirektoratet for endelig behandling av spørsmålet om oppretting av forskningsbiobank.

Med vennlig hilsen

Kristian Hagestad
Fylkeslege cand.med., spes. i samf.med
Leder

Jørgen Hardang
Sekretær

Kopi: Sosial- og helsedirektoratet

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Nettadresse: www.etikkom.no

Dato: 2.5.08
Deres ref.:
Vår ref.: S-07468a

S-07468a Astma og allergi hos Olympiere [1.2007.2840]

Vi viser til skjema for protokolltillegg og endringer datert 22.4.08.

Komiteen godkjenner at prosjektet videreføres med de endringer som er beskrevet i skjema for protokolltillegg og endringer forutsatt at informasjonsskriv med samtykkeerklæring ettersendes og finnes tilfredsstillende.

Med vennlig hilsen

Kristian Hagestad
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Leder

Jørgen Hardang
Sekretær

SÃO JOÃO

A CA
DC

· Hospital São João ·
Paulo Bettencourt
Adjunto da Direcção Clínica

Exma. Senhora

Dra. Margarida Tavares

Directora Clínica

Centro Hospitalar de São João

Assunto: Projectos de Investigação

Para submissão a apreciação pelo Conselho de Administração, junto remeto a V. Exa o seguinte projecto de investigação com pareceres finais da CES e da Unidade de Investigação:

n_id_ cic	n_id_ces	título	investigador principal
174/12	58/12	Mechanisms of airway damage in elite swimmers	Mariana Couto

Porto, 25 de Julho de 2012

Com os meus melhores cumprimentos,

A Secretária da Unidade de Investigação

(Sandra Pereira)

AUTORIZADO

CONSELHO DE ADMINISTRAÇÃO @ REUNIÃO DE 27 JUL 2012

Presidente do Conselho de Administração

(Prof. Doutor António Ferreira)

Directora Clínica	Enfermeira Directora	Vogal Executivo	Vogal Executivo
(Dra Margarida Tavares)	(Enfermeira Eurídice Portela)	(Dr. [illegible] Barros)	(Dr. João Oliveira)

Parecer

Título do Projecto: "Programa doutoral em medicina – mechanisms of airway damage in elite swimmers

Nome do Investigador Principal: Mariana Couto

Serviço onde decorrerá o Estudo: No Serviço de Imunoalergologia / laboratório de Imunoalergologia do C.H.S.João

Objectivo do Estudo:

Contribuir para o esclarecimento dos mecanismos que determinarão a ocorrência de asma induzida pelo exercício, particularmente em atletas de elite praticantes de natação e desportos de inverno.

Concepção e Pertinência do estudo:

Com este projeto, a investigadora pretende procurar uma resposta para o risco aumentado de asma e doenças alérgicas em desportistas de elite, sobretudo nos que praticam natação e desportos de inverno. Especificamente, pretende avaliar a perceção da dispneia, conhecer os efeitos a longo prazo da prática regular da natação de competição, identificar as variáveis relacionáveis com o controlo da asma, verificar qual o papel da inflamação neurogénica na etiopatogenia da asma dos nadadores e quais os mediadores de dano e angiogénese nas vias aéreas.

Serão, para o efeito, recrutados atletas do Futebol Clube do Porto, de maior idade. Estes atletas já participaram em anterior investigação, tendo já, pois, conhecimento do tipo de avaliações a que serão submetidos de novo: testes cutâneos para alergia, avaliação da inflamação pulmonar por determinação de óxido nítrico no ar exalado e esputo induzido, avaliação da função pulmonar e reversibilidade e, bem assim, avaliação da hiperreatividade brônquica na prova da metacolina.

À realização destas avaliações estão identificados potenciais efeitos adversos, a forma de os identificar e classificar. Em resposta a questões colocadas à investigadora no parecer inicialmente elaborado, foram clarificadas e afirmadas as condições de segurança em que toda a investigação será feita e, de tal, dada adequada informação aos participantes (foi corrigido o documento informativo para obtenção de consentimento!).

Benefício/risco: Os participantes poderão beneficiar do diagnóstico e tratamento da asma e outras doenças alérgicas. De acordo com os esclarecimentos dados pela investigadora, os riscos, descritos no documento informativo, são aceitáveis.

Respeito pela liberdade e autonomia do sujeito de ensaio: Será solicitado e obtido consentimento aos representantes das crianças em cujas consultas se adequará a participação do investigador.

Confidencialidade dos dados: São dadas garantias de confidencialidade. Por indicação da CES, os questionários a utilizar foram anonimizados.

Elo de ligação: NA

Indemnização por danos: Não previstos

Continuação do tratamento: NA

Propriedade dos dados: Não indicado

Curriculum do investigador: Adequado ao perfil da investigação.

Data previsível da conclusão do estudo: 2015

Conclusão:

1. Este projeto de investigação tem objetivos que justificam a sua realização. Todavia, houve questões metodológicas que receberam reparo da CES e que foram adequadamente corrigidas.
2. Tal como decorre do parecer inicialmente aprovado, no seu atual desenho metodológico, a CES não levanta objeções à sua realização.

Porto e H.S.João, 2012-07-16

O Presidente da CES

Doutor Filipe Almeida

CES

COMISSÃO DE ÉTICA PARA A SAÚDE

8. TERMO DE RESPONSABILIDADE

Eu, abaixo-assinado, Mariana Couto, na qualidade de Investigador Principal, declaro por minha honra que as informações prestadas neste questionário são verdadeiras. Mais declaro que, durante o estudo, serão respeitadas as recomendações constantes da Declaração de Helsínquia (com as emendas de Tóquio 1975, Veneza 1983, Hong-Kong 1989, Somerset West 1996 e Edimburgo 2000) e da Organização Mundial da Saúde, no que se refere à experimentação que envolve seres humanos.

Porto, 29 / Fevereiro / 2012

O Investigador Principal

A Comissão de Ética para a Saúde tendo aprovado o parecer do Relator, aguarda que o Investigador/Promotor esclareça as questões nele enunciadas para que possa emitir parecer definitivo.

2012-03-29

Presidente da Comissão de Ét...

PARECER DA COMISSÃO DE ÉTICA PARA A SAÚDE DO HOSPITAL DE S. JOÃO

emitido na reunião plenária da CES de ___/_____/_____

Considerado por forma como cabais os esclarecimentos e as correcções introduzidas

A Comissão de Ética para a Saúde APROVA por unanimidade o parecer do Relator, pelo que nada tem a opor à realização deste projecto de investigação.

Prof. Doutor Filipe Alma... Presidente da Comissão de Ét...

2012-07-16

Appendix II

The AQUA-questionnaire

3657

Patient ID ☐☐ - ☐☐☐

Centre ID ☐☐

Modified AQUA$_{2008}$

Questionnaire for assessment of asthma, allergy and other repiratory disorders for athletes participating in the Summer Olympic Games in Beijing August 2008

Country _____

Date of birth (Day Month Year) ☐☐.☐☐.☐☐☐☐

Age (years): ☐☐

Gender: ☐ Male ☐ Female

Weight (kg): ☐☐☐

Height (cm): ☐☐☐

Type of sport _____

Sports Association _____

Club _____

1. Have you previously participated in other types of sports on a competitive level? ☐ Yes ☐ No

1b. Which other kind of sport did you practice? _____

2. How many times a week do you exercise? ☐ 3 ☐ More than 3 ☐ Daily

3. Every training session usually lasts:
   - ☐ Less than 2 hours
   - ☐ 2-3 hours
   - ☐ More than 3 hours

4. Are you training mainly: ☐ Outdoor ☐ Indoor ☐ Both

5. Did any doctor diagnose you with any of these allergic diseases?
   - ☐ Asthma
   - ☐ Allergic rhinitis (Hayfever)
   - ☐ Allergic conjunctivitis (with eye symptoms)
   - ☐ Urticaria (hives)
   - ☐ Atopic eczema
   - ☐ Drug allergy
   - ☐ Food allergy
   - ☐ Insect venom allergy (bee, wasp)
   - ☐ Anaphylaxis (Allergic shock)

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Patient ID ☐☐ - ☐☐☐

6. Do you suspect that you suffer from allergy, independently of any medical diagnosis ?

☐ Yes ☐ No

7. Have you ever used anti-allergic or anti-asthma drugs ? ☐ Yes ☐ No

7b. If yes, which?

- ☐ Antihistamins
- ☐ Corticosteroids
- ☐ Bronchodilators
- ☐ Laukotrien antagonists (singulair)
- ☐ Allergy vaccines

8. Is there any allergic subject in your family? ☐ Yes ☐ No

8b. If yes, who?

- ☐ Mother
- ☐ Father
- ☐ Sibling(s) including half siblings
- ☐ Other relatives
- ☐ Children

9. Do you often have red eyes with tears and itching? ☐ Yes ☐ No

10. Do you often have runny, itchy nose (apart from colds): ☐ Yes ☐ No

11. Have you ever felt tightness in your chest and/or wheeze? ☐ Yes ☐ No

12. Have you ever had itchy skin eruptions? ☐ Yes ☐ No

13. Have you ever had severe allergic or anaphylactic reactions? ☐ Yes ☐ No

14. Have you ever had shortness of breath, cough and/or itching of the throat during or following exercise? ☐ Yes ☐ No

14b. If yes, you have more difficulties:

- ☐ At the beginning of the training session
- ☐ At the end of the training session
- ☐ During the whole training session

15. If you have suffered from any of the above, did these symptoms occur:

- ☐ Mainly outdoor
- ☐ Mainly indoor
- ☐ Indoor and outdoor equally
- ☐ Mainly in spring
- ☐ Mainly in cold or humid conditions
- ☐ All year around
- ☐ Independently of any environmental conditions

3657

Patient ID ☐☐ - ☐☐☐

16. Have you ever had allergic reactions to foods? ☐ Yes ☐ No

16b. If yes, do you remember to which food? _____

17. Have you ever had allergic reactions to drugs? ☐ Yes ☐ No

17b. If yes, do you remember to which drug? _____

18. Do you know that some drugs for allergic and respiratory diseases are prohibited or under restrictions by the World Anti-Doping Agency (WADA)? ☐ Yes ☐ No

18b. If yes, tick which substances, you think are included in this category:

- ☐ Antihistamines
- ☐ Bronchodilators
- ☐ Vasoconstrictors
- ☐ Topical corticosteroids (Nasal inhalers, eye droplets, dermatological preparations)
- ☐ Inhaled corticosteroids
- ☐ Injected or oral corticosteroids

19a. Do you think that anti-allergic and/or respiratory drugs may:

☐ Reduce performance ☐ Improve performance ☐ Don't affect performance

19b. Do you think that anti-allergic and/or respiratory drugs may be in conflict with anti-doping regulations? ☐ Yes ☐ No

20. Have you used more than three courses of any of these drugs during the last year? ☐ Yes ☐ No

20.b.. If yes, tick which category of drugs you did use:

- ☐ Antibiotics
- ☐ Anti inflammatory drugs
- ☐ Pain reducing drugs
- ☐ Drugs for reducing fever
- ☐ Others, which....

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Patient ID ☐☐ - ☐☐☐

21. Have you used any other (except anti-asthma/anti-allergic) drug during the last week?

☐ Yes ☐ No

21 b. If yes, which drug? _____

22. Do you frequently suffer from upper respiratory infections (pharyngitis, colds, otitis media, tonsillitis, laryngitis) or fever?

☐ Yes ☐ No

22 b. If yes, are these infections more frequent during periods when you train more often than usual or during overtraining periods?

☐ Yes ☐ No

23. Have you suffered from recurrent labial herpes?

☐ Never
☐ 1-3 times
☐ More than 3 times

24. How many times during the last year were you unable to train because of infections?

☐ Never
☐ 1-3 times
☐ More than 3 times

25. If you have respiratory symptoms, which?

☐ Episodes of heavy breathing
☐ Wheeze
☐ Cough
☐ Phlegm, expectorate

26.Does this occur?

a. During exercise / training / competition: ☐ Yes ☐ No

b. During colds ☐ Yes ☐ No

c, After contact with animals, pollens, others: ☐ Yes ☐ No

27. With respiratory symptoms and dyspnoea related to exercise, when and how?

a. During maximum exercise ☐ Yes ☐ No

b. After the exercise: ☐ Yes ☐ No

c. In the afternoon, after training and/or competition: ☐ Yes ☐ No


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Patient ID ☐☐ - ☐☐☐

28. When you have these respiratory symptoms?

a. Is it difficult to inhale ☐ Yes ☐ No

b. Is it difficult to exhale ☐ Yes ☐ No

c. Both: ☐ Yes ☐ No

29. Do the respiratory symptoms / dyspnoea occur?

☐ Outdoors
☐ Indoors
☐ Both outdoors and indoors

30. How often do you have heavy breathing?

☐ Daily
☐ Several times a week
☐ Weekly
☐ Monthly
☐ More rarely

31. Does your respiratory symptoms increase with simultaneously?

☐ Low temperatures, cold air inhaled
☐ Fog

32. Do the respiratory symptoms have impact on your sports performance? ☐ Yes ☐ No

33. Do you have symptoms from eyes or nose? ☐ Yes ☐ No

34 a. Do you smoke? ☐ Yes ☐ No

34 b. If yes, how many cigarettes a day?

☐ Less than 5
☐ 5-20
☐ More than 20

35. Do you use snus? ☐ Yes ☐ No

36. Do you use any foods supplements (vitamins, amino acids, creatine)? ☐ Yes ☐ No

Appendix III

Informed consent Study I

Forespørsel om å delta i en forskningsstudie:

«Er utholdenhetsidrett skadelig for luftveiene?»

Bakgrunn og hensikt
Forekomsten av astma er høyere blant idrettsutøvere enn hos personer som ikke driver idrett, spesielt blant utøvere innen utholdenhetsidretter som utføres i kulde eller i svømmehaller. Vi vet lite om årsakene til dette. Hensikten med studien er derfor å undersøke om systematisk utholdenhetstrening kan føre til skader på luftveiene som over tid kan lede til astma. Vi vil spesielt undersøke sammenheng mellom høyintensiv trening og betennelsesprosesser i luftveiene, på nervesystemene i luftveiene og lungefunksjonen.

Du blir forespurt om å delta fordi du er:
- **idrettsutøver innen utholdenhetsidrett med astma**
- **idrettsutøver innen utholdenhetsidrett uten astma**
- **er frisk og ikke driver konkurranseidrett (kontrollgruppe)**

Vi søker kvinner og menn i alderen 16-35 år. Idrettsutøvere må konkurrere på et høyt nasjonalt eller internasjonalt nivå og trene mer en 10 timer per uke. Kontrollgruppen kan ikke drive konkurranseidrett og må trene mindre enn 5 timer per uke.

Hva innebærer studien?
Som forsøksperson vil du bli innkalt til to undersøkelser i løpet av tre uker på Norges idrettshøgskole i Oslo. Hver undersøkelse vil vare ca. 2 timer og må foregå på separate dager med minst 24 timer mellom.

Det vil bli utført medisinske undersøkelser inkludert lungefunksjonsundersøkelser, allergitest, måling av ulike betennelsesmarkører i kondensat fra utpust og oppsamlet sputum (slim) fra lungene. Vil vil også måle pupillenes reaksjon på lys og reaktivitet i luftveiene. Du vil utføre en 4-sekunders sykkeltest og fylle ut et spørreskjema relatert til astma, allergi, fysisk aktivitet og idrett. Vi vil også gjennomføre et kort intervju med spørsmål om sykehistorie og medisinbruk. På dag 2 må du ta med en morgenurinprøve og en spyttprøve som du tar hjemme om morgenen, og vi vil ta en blodprøve. Undersøkelsene vil bli gjort av doktorgradsstipendiat Julie Stang og masterstudenter i samarbeid med lege. Se **Kapittel A** for detaljert beskrivelse av undersøkelsene.

Mulige fordeler og ulemper
Det foreligger ingen umiddelbare fordeler for deg ved å delta, men du vil få en grundig lungefysiologisk undersøkelse og en allergi test. Målingene som utføres er ufarlige og medfører ingen spesiell risiko. Oppsamling av sputum kan være ubehagelig og medfører at du hoster opp slim. Målingen av luftveienes ømfintlighet kan føre til kortvarig og forbigående tung pust som vil reverseres med astmamedisin etter testen.

Hva skjer med prøvene og informasjonen om deg?
Noen av prøvesvarene, som allergitesten og lungefunksjon, formidles direkte til deg på undersøkelsesdagen. Andre undersøkelser vil du ikke få svar på, fordi de vil bli analysert på laboratorier med høy vitenskapelig kompetanse i Norge, Europa, USA eller andre land i henhold til det mest velegnede laboratoriet for den angjeldende analyse. Disse inngår i forskning og har usikker klinisk betydning for enkeltindivider. Informasjonen som registreres om deg er anonym og vil kun brukes slik som beskrevet i hensikten med studien.

Frivillig deltakelse
Dersom du ønsker å delta, undertegner du samtykkeerklæringen på siste side. Det er frivillig å delta i studien og du kan når som helst og uten å oppgi noen grunn trekke ditt samtykke til å delta i studien.

Prosjektadministrasjon
Studien foregår i regi av Oslo Universitetssykehus i samarbeid med idrettsmedisinsk seksjon på Norges idrettshøgskole. Ansvarlig for prosjektet er professor Kai-Håkon Carlsen ved Universitetet i Oslo, Oslo Universitetssykehus og Norges idrettshøgskole.

Ytterligere informasjon om biobank, personvern og dine rettigheter finnes i Kapittel B

Har du spørsmål?
Kontaktpersoner:
Julie Stang, tlf: 23 26 24 01/98 41 14 40 eller epost: julie.stang@nih.no
Kai-Håkon Carlsen, tlf: 22 13 65 22 / 92 01 70 26 eller epost: k.h.carlsen@medisin.uio.no

Kapittel A: Utdypende forklaring for hva studien innebærer

Hvis du sier ja til å delta i studien, vil du få følgene informasjon fra oss:

- Brev om oppmøte og informasjon om undersøkelsene.
- På første undersøkelsesdag vil du få med deg 2 prøveglass hjem for å samle morgenurin og for spyttprøven. Dette tar du med til dag 2.
- Etter dag 2 vil du få et kort sammendrag av hva slags undersøkelse du har gjennomført og resultatene av disse undersøkelsene fra lege.

Undersøkelsene:
Når du kommer til undersøkelse kan du ikke ha vært syk de siste 3 ukene på forhånd (forkjølet, influensa, infeksjon el.). Dersom du er syk må vi utsette undersøkelsen til det har gått 3 uker.

Du kan ikke være under påvirkning av luftveisutvidende medikamenter eller allergimedisiner. Dette betyr at følgene medikamenter skal ikke inntaes:

Samme dag	Inhalasjonspreparater av kortison: Pulmicort®, Flutide®, Aerobec®, Becotide®, Alvesco®, Astmanex®
8 timer før undersøkelse	Ventoline®, Salbuvent®, Inspiryl®, Bricanyl®, Airomir® og Lomudal til inhalasjon
12 timer før undersøkelse	Atrovent® til inhalasjon
24 timer før undersøkelse	Dymista®
3 døgn før undersøkelse	Serevent®, Seretide®, Oxis®, Symbicort®, Singulair®, Flutiform® og Teophylline preparater (TheoDur®, Nuelin deport®)
7 døgn før undersøkelse	Antihistaminer: Phenamin®, Aereus®, Zyrtex®, Cetirizine®, Reactine®, Xyzal®, Clarityn®, Versal®, Loratadine®, Kestine®, Telfast®, Vallergan®

Oversikt over testdagene:

Dag 1. Ca 2 timer		**Dag 2. Ca 1.5 timer**
1. Ekshalert NO 2. Lungefunksjon 3. Allergitest 4. Intervju med spørreskjema 5. Pupillometri og 4 sek. sykkeltest 6. Blodprøve 7. Kondensat fra utpust (EBC) 8. Metakolin inhalasjonstest	**Min. 24 timer**	1. Spyttkortisol og urinprøve (tas hjemme og medbringes) 2. Ekshalert NO 3. Pupillometri og 4 sek. sykkeltest 4. Lungefunksjon 5. Lungevolum, diffusjonskapasitet 6. Indusert sputum (Prøve av slim hostet opp fra lungene)

Hva som gjøres:

1. Ekshalert nitrogenoksyd (NO) måles ved at du trekker pusten så dypt du kan og så puster ut med en jevn luftstrøm i 10 sekunder. NO er en markør på grad av betennelse i nedre luftveier. Du vil gjennomføre 2-3 forsøk.

2. Lungefunksjonen din måles ved at du trekker pusten så dypt du kan før du blåser ut hardt, fort og lenge gjennom et munnstykke. Du vil gjennomføre 2-3 forsøk og hvert forsøk varer ca 15 sekunder.

3. Det vil bli utført en prikktest for å vurdere allergi. De mest vanlige allergenene (pollen, dyrehår, muggsopp og husstøvmidd) er konsentrert i en liten dråpe saltvann (ca 12 ulike dråper) som legges

på underarmen og prikkes så vidt under huden med en lansett. Testen tar ca 5 minutter å utføre og resultatet avleses etter 15 min.

4. Du vil bli bedt om å svare på et spørreskjema med spørsmål ang. astma og allergi.

5. Vi undersøker aktivitet i det parasympatiske nervesystemet ved å måle endringer i hjertefrekvens ved bruk av en avansert pulsklokke under en svært kort sykkeltest. Under testen blir du bedt om å holde pusten i fire sekunder før du tråkker så raskt du kan i fire sekunder på en ergometersykkel. Vil vil også måle hvor raskt pupillen din trekker seg sammen etter et lysglimt. Dette kalles pupillometri og testen tar kun noen sekunder.

6. Blodprøve, urinprøve og spyttprøve samles for å analysere på stoffer relatert til astma og allergi.

7. Vi vil samle opp kondensat fra luft du puster ut for analyse. Du skal da sitte i ro og puste helt normalt, inn gjennom nesen og ut i et munnstykke i 15 minutter. Dette kan gi informasjon om betennelser i luftveiene.

8. Du gjennomfører en metakolin inhalasjonstest for å bestemme reaktiviteten i luftveiene. Dette gjøres ved å måle lungefunksjonen før og etter inhalasjoner med et stoff som virker irriterende på luftveiene (metakolin). Du puster inn metakolin i økende doser, inntil lungefunksjonen faller 20%. Avhengig av grad av reaktivitet vil testen ta mellom 5 og 20 minutter. Når du er ferdig får du astmamedisin (Ventoline®) for å åpne luftveiene helt igjen. Denne undersøkelsen kan gi en forbigående følelse av tetthet i brystet, men det er svært lite uttalt. En lungefunksjonsmåling gjennomføres 15 min etter inhalasjon av astmamedisin.

9. På dag 2 vil vi måle lungevolumer ved 2 ulike målemetoder, samt diffusjonskapasitet og motstand i luftveiene dine. Dette gjøres ved enkle pustetester som vil samlet ta ca. 15 minutter.

10. Det vil taes en prøve av slim fra luftveiene dine. Dette kalles indusert sputum. Prosedyren går ut på en inhalasjon av inhalasjon av saltvann som gjør «hoster» opp slim som vil bli analysert for innhold av inflammatoriske celler og epitelskade. Varighet på en slik prosedyre vil være på mellom 20-45 minutter og vil avhenge fra person til person.

Kapittel B: Personvern, biobank, økonomi og forsikring

Personvern og frivillig deltakelse
All informasjon som samles inn i løpet av prosjektet er konfidensielle opplysninger som lagres forskriftmessig. Opplysninger og prøvesvar vil bli behandlet uten navn, fødselsnummer eller andre direkte gjenkjennende opplysninger ved at hver forsøksperson får et forsøksnummer. Koblingen mellom navn og forsøksnummer blir oppbevart i en lukket forskningsserver ved Oslo Universitetssykehus. Kun autorisert personell knyttet til prosjektet har innsyn i resultatene vedrørende den enkelte forsøksperson.

Hvis du trekker deg fra studien vil det ikke få noen konsekvenser for din videre behandling, eller forholdet til OUS eller Norges idrettshøgskole. Du har også rett til innsyn i data registrert om deg.

Sikkerhet
Undersøkelser som inngår i studien er vanlig benyttet klinisk praksis. Behandling for eventuelt respirasjonsbesvær vil kunne gis umiddelbart og det vil alltid være en erfaren lege tilstede ved undersøkelsene.

Etikk og biobank
Studien er godkjent av Regional Komité for medisinsk og helsefaglig forskningsetikk (REK)-Øst. Hvis du sier ja til å delta i studien, gir du også samtykke til at det biologiske materialet og analyseresultater inngår i en forskningsbiobank ved Oslo Universitetssykehus. Du gir du også ditt samtykke til at prøver kan utleveres til samarbeidende institusjoner for analyse, etter gjeldende retningslinjer og bli sendt til andre land, både i og utenfor Europa. Wenche Reed er ansvarshavende for biobanken, som planlegges å vare til 2028. Etter dette vil all informasjon bli anonymisert etter interne retningslinjer, dersom ikke endret samtykke foreligger.

Videre behandling av forsøksresultatene
Resultatene fra studien vil bli vitenskapelig behandlet og publiseres i internasjonale og nasjonale tidsskrifter og rapporter.

Rett til innsyn og sletting av opplysninger om barnet og sletting av prøver
Hvis du sier ja til at å delta i studien, har du rett til å få innsyn i hvilke opplysninger som er registrert om deg. Du har videre rett til å få korrigert eventuelle feil i de opplysningene vi har registrert. Dersom du trekker deg fra studien, kan du kreve å få slettet innsamlede prøver og opplysninger, med mindre opplysningene allerede er inngått i analyser eller brukt i vitenskapelige publikasjoner.

Økonomi
Studien og biobanken er finansiert gjennom forskningsmidler fra Norges idrettshøgskole og forskningsgruppen ORAACLE ved Oslo Universitetssykehus. Studien er en del av et doktorgradsprosjekt ved Norges idrettshøgskole.

Forsikring
NIH er statlige institusjon og er derfor selvassurandør i forhold til studien.

Informasjon om utfallet av studien
Resultatene fra studien vil bli gjort offentlig tilgjengelig gjennom artikler og eventuelt rapporter. Det er planlagt å omtale studien i Allergi i Praksis som utgis av Norges Astma og Allergiforbund.

Samtykke

Jeg har lest informasjonsskrivet om Forespørsel om å delta i en forskningsstudie: «Er utholdenhetsidrett skadelig for luftveiene?».

Jeg gir min tilslutning til deltagelse i undersøkelsen. Jeg er kjent med at jeg når som helst kan trekke meg fra prosjektet uten å måtte oppgi grunn for det. Jeg er klar over at de innsamlede data utelukkende brukes til forskning.

Forsøkspersonens navn: _____

Jeg nåes på telefon (dagtid): _____

Epostadresse: _____

Dato: _____ Underskrift: _____

For foresatte dersom forsøkspersonen er under 18 år:
Foresatte skriver under i tillegg til forsøkspersonen.

Dato: _____ Underskrift foresatte: _____