THE MULTIMORBIDITY OF ASTHMA AND RHINITIS:

FROM EPIDEMIOLOGIC DATA TO MOLECULAR TRAITS

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PhD Dissertation in Medicine

Sub-specialization Clinical Research

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To those who live the multimorbidity of asthma and rhinitis

Every day

This dissertation reflects the joint work and commitment of several great minds with whom I had the privilege to be with, to discuss and to learn. They have taught me, not only with words but also with gestures, not only knowledge and specific scientific skills, but especially curiosity, rigor, will and creativity to face difficulties as new opportunities, and resilience and strength to achieve our goals.

With a common goal, this dissertation has gathered multidisciplinary teams of researchers, with distinct backgrounds and competences, addressing complementary specific aims and congregating different methodologies, from already ongoing, implemented projects to the design and setting of new research. This work has embraced the development of research ideas and questions, study design and responsibility for its approvals and funding, protocols writing, cost center management, materials purchasing and the setting of a research-friendly environment to implement the experimental work in the core of clinical practice, welcoming participants with respect and rigor. It also enclosed the collecting and handling of biological samples, allergy, inflammatory and respiratory function testing, data extraction and organization, conducting and critically interpreting statistical analyses and bringing our work into the scientific community with writing, submitting and presenting abstracts and manuscripts, answering reviewers' comments, simplifying messages to the general public and, at the end, never stopping questioning towards an ever better proof. This entire beginning journey, with both its backwards and forwards, has been of inestimable value to me.

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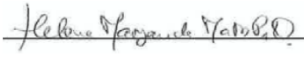
For my parents and grandparents, my warmest thanks for the firm groundings for life, for being with me and letting me fly. For Alexandre, Gabriela and Mário, for us, my true reasons of living, my unconditional love.

Lisbon, 30th April 2018



"Imagination is more important than knowledge. For knowledge is limited to all we now know and understand, while imagination embraces the entire world, and all there ever will be to know and understand."

Albert Einstein
(1879-1955)

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Abbreviations

ACO – Asthma-Chronic obstructive pulmonary disease Overlap

AirPROM – Airway disease PRedicting Outcomes through patient specific computational Modelling

ARIA – Allergic Rhinitis and its Impact on Asthma

CARAT – Control of Allergic Rhinitis and Asthma Test

CARATkids – Control of Allergic Rhinitis and Asthma Test for children

CI – Confidence Interval

COPD – Chronic Obstructive Pulmonary Disease

EARIP – European Asthma Research and Innovation Partnership

EBC – Exhaled Breath Condensate

ECRHS – European Community Respiratory Health Survey

e.g. – *exempli gratia* ("for example")

FeNO – Fractional exhaled Nitric Oxide

FEV_1 – Forced Expiratory Volume in one second

GA^2LEN – Global Allergy and Asthma Network of Excellence

GARD – Global Alliance against chronic Respiratory Diseases

GINA – Global INitiative for Asthma

ICAR – Control and burden of asthma and rhinitis study (Impacto e Controlo da Asma e da Rinite)

i.e. – *id est* ("that is")

IgE – immunoglobulin E

IL – interleukin

INAsma – Portuguese national asthma survey (Inquérito Nacional sobre Asma)

ISAAC – International Study of Asthma and Allergy in Childhood

MeDALL – Mechanisms of the Development of ALLergy

n.b. – *nota bene* ("note well")

NMR – Nuclear Magnetic Resonance

OR – Odds Ratio

PCA – Principal Component Analysis

PEF – Peak Expiratory Flow

PNIF – Peak Nasal Inspiratory Flow

Th – T helper

U-BIOPRED – Unbiased BIOmarkers in PREDiction of respiratory disease outcomes

Abstract

Introduction and Aims: Worldwide and across all age groups, asthma affects the lives of several hundred million people. In spite of the advances over the last decades, asthma and its multimorbidity continue to impart a significant onus on individuals with the disease, their families and society and also on health economies. A high number of unmet needs remain to be resolved, related to gaps in current scientific knowledge covering many aspects of asthma, from epidemiology and pathophysiology to patient care. The main objective of this dissertation was to contribute to address some of these existing unmet needs in asthma and its link with rhinitis. In particular, the original work aimed to (1) estimate nationwide asthma prevalence and analyze its association with rhinitis in particularly vulnerable and internationally data-lacking population groups – the children and the elderly; (2) unveil features for an early recognition of asthma, identifying multidimensional "hypothesis-free" early childhood wheezing clinical phenotypes related to asthma persistence in adolescence; (3) analyze the association between nasal and lower airway function, together with the subjective evaluation of allergic rhinitis and asthma concurrent control in children; (4) explore innovative strategies to uncover "unbiased" differentiating metabolic features of childhood allergic rhinitis and asthma multimorbidity in non-invasively collected samples.

Methods: This dissertation was based on three types of studies:

1. Cross-sectional, population-based, nationwide surveys of citizens living in Portugal, applied by interview using standardized procedures, to collect epidemiological data related to asthma and rhinitis and to analyze the association between these two conditions. For the pediatric study, data from all individuals aged below 18 years who participated in the INAsma study (population-based, all-age, nationwide telephone interview study to estimate asthma prevalence in Portugal) was analyzed. The elderly-targeted study was originally designed to estimate rhinitis prevalence in individuals aged 65 years or above living in mainland Portugal and the data was collected by direct face-to-face interview;
2. Prospective cohort study of children aged below 7 years with recurrent wheezing, systematically evaluated at specific time-points, up to 13 years of follow-up. Multivariable logistic regression models for persistent asthma in adolescence were developed based on questionnaires and skin prick tests data. Clinical phenotypes were identified by cluster analysis of variables selected with the logistic regression analysis, and compared for predicting asthma prevalence, use of control treatments and lung function in childhood and adolescence;
3. Cross-sectional, case-control study of school-aged children with allergic rhinitis and asthma multimorbidity and healthy children (matched for age and gender), evaluated with respect to:
    a. Respiratory functional laboratorial assessments, i.e., sequential assessments of peak nasal inspiratory flow (PNIF) before and after nasal decongestion and spirometry with bronchodilation test. The Control of Allergic Rhinitis and Asthma Test for children (CARATkids) was used for these diseases concurrent subjective control evaluation. Associations between objective and subjective scores were investigated by multiple linear regression models.
    b. Analytical laboratorial study using untargeted metabolomics analysis by nuclear magnetic resonance (NMR) spectroscopy of urine and saliva samples collected from each child. Spectroscopic and clinical data were subjected to statistical analysis including multivariable and univariable approaches. Additionally, exhaled breath condensate (EBC) samples were collected from volunteers, together with room air samples, which were analyzed by NMR spectroscopy. The resulting spectra were compared.

Results: The estimated prevalence of current asthma in children was 8.4% (95% confidence interval (CI) 6.6%-10.7%). The prevalence of rhinitis and of physician-diagnosed asthma in the elderly were estimated to be 29.8% (95%CI 28.4%-31.3%) and 10.9% (95%CI 9.9%-11.9%), respectively. A strong association between asthma and rhinitis at the population-level was found both in children (odds-ratio (OR) 5.2, 95%CI 3.1-8.9) and in the elderly (OR varying from 8.3, 95%CI 6.1-11.4 in mild intermittent rhinitis to 39.9, 95%CI 27.5-58.0 in moderate-severe persistent rhinitis).

In the cohort study, atopy and rhinitis at preschool-age were independent risk factors for asthma persistence in adolescence (OR 11.8, 95%CI 4.0-34.6, and OR 10.4, 95%CI 3.7-29.1, respectively). Three distinct early childhood wheezing phenotypes were identified, which were predictive of asthma persistence, use of control treatments and lung function in school-age and adolescence. Multimorbidity, particularly rhinitis, with or without associated atopy, tended to predict a worse prognosis.

In the nasal and lung function study, baseline and decongested PNIF correlated with baseline and post-bronchodilation peak expiratory flow (PEF) and forced expiratory volume in one second (FEV_1) in school-aged children, observed independently of rhinitis and asthma diagnosis. The best linear regression model for PNIF included the variables PEF, age and gender. In children with allergic rhinitis and asthma, no association was found between PNIF and CARATkids scores, except for nasal obstruction self-report.

Untargeted metabolomics analysis of saliva and urine samples revealed a subset of the spectral areas significantly different in the children with allergic rhinitis and asthma, compared to healthy controls. Some metabolites contributing to these variables were identified: arginine, taurine, citrate and aspartate (in saliva), and quinolinate, butyrate, pantothenate, gluconate, pseudouridine and lysine (in urine). Urinary quinolinate, butyrate and pantothenate concentrations correlated with spirometric parameters, while quinolinate, gluconate and pseudouridine concentrations correlated with exhaled nitric oxide (FeNO) levels. Urinary quinolinate and salivary citrate and aspartate were associated with multiple allergenic sensitization. The EBC metabolic profile was found to be highly comparable to the ambient air spectral composition.

Discussion and Conclusions: These were the first population-based nationwide epidemiologic studies reporting asthma symptoms prevalence among all pediatric ages, and rhinitis prevalence, its classification and association with asthma in the elderly. Our results further support that asthma is a common disease in children and the elderly, frequently associated with rhinitis. In early childhood, the presence of multimorbidity, particularly rhinitis with or without associated atopy, tended to predict a worse prognosis of recurrent wheezing regarding asthma persistence and impaired lung function in later childhood and adolescence. These results reinforce the need for a global, integrated care pathway in asthma and rhinitis, since early ages. In this integrated assessment, PNIF may provide complementary objective information to subjective concurrent control assessment of allergic rhinitis and asthma in school-aged children. The results suggested that PEF values should ideally be considered, besides age and gender, when interpreting PNIF values in this age group. Exploratory metabolomics revealed differentiating subsets of NMR spectral features in saliva and urine associated with allergic rhinitis and asthma multimorbidity in children, generating hypotheses to be further analyzed. The results obtained in the EBC metabolic profile analysis reinforced the importance of ambient air controls during samples collection and the need for analytical procedures to distinguish exogenously originated metabolites in EBC. In summary, the results presented in this dissertation added compelling information for an integrated, global assessment of asthma together with rhinitis, in clinical practice and in research. We foresee the clinical general application of nasal and lung function evaluation in a global airways assessment strategy. The differentiating subsets of metabolites found in exploratory metabolomics analysis stimulate further studies in order to validate our findings, followed by the identification of molecules/metabolic pathways involved, its role in allergic rhinitis and asthma pathophysiology and ultimately the potential as (novel) therapeutic targets.

Abstract in Portuguese

Introdução e Objetivos: A asma afeta a vida de várias centenas de milhões de pessoas de todas as idades, em todo o mundo. Apesar dos avanços nas últimas décadas, a asma e a sua inerente multimorbilidade permanecem um ónus significativo para as pessoas com a doença, para as suas famílias e para a sociedade e economia da saúde. Um número elevado de questões permanece por responder, abrangendo vários aspetos da doença, relacionados com lacunas no conhecimento científico atual, desde a epidemiologia à fisiopatologia e aos cuidados prestados à pessoa com asma. O objetivo principal desta dissertação foi contribuir para a abordagem de algumas destas questões relativas à asma e a sua associação com a rinite. Em particular, os trabalhos originais visavam: (1) estimar a prevalência de asma em Portugal e analisar a sua associação com a rinite em grupos populacionais particularmente vulneráveis e sobre os quais há carência de dados a nível internacional - crianças e idosos; (2) identificar características para um reconhecimento precoce de asma, através de fenótipos clínicos multidimensionais de sibilância recorrente em idade pré-escolar, estabelecidos “sem hipóteses pré-definidas" e relacionados com a persistência de asma na adolescência; (3) analisar a associação entre parâmetros funcionais respiratórios das vias aéreas superiores e inferiores, em conjunto com a avaliação subjetiva do controlo da rinite alérgica e da asma, em crianças em idade escolar; (4) explorar estratégias inovadoras para identificar características metabólicas associadas ao fenótipo de asma e rinite alérgica em crianças, em amostras colhidas de forma não invasiva.

Métodos: Esta dissertação baseou-se em três tipos de estudos:

1. Estudos transversais, baseados na população nacional, de cidadãos que viviam em Portugal, tendo sido aplicados questionários por entrevista, usando procedimentos padronizados, para a obtenção de dados epidemiológicos relativos à asma e à rinite. Para o estudo pediátrico, foram analisados os dados de todos os indivíduos com idade inferior a 18 anos que participaram no estudo INAsma (estudo por entrevista telefónica, de base populacional, nacional, para estimar a prevalência de asma em Portugal). O estudo dirigido aos idosos foi desenhado para estimar a prevalência de rinite em adultos com 65 anos ou mais, residentes em Portugal continental, tendo sido os dados colhidos por entrevista direta;

2. Estudo prospetivo de coorte de crianças com idade inferior a 7 anos com sibilância recorrente, avaliadas sistematicamente em pontos de tempo específicos, até 13 anos de seguimento. Foram desenvolvidos modelos de regressão logística multivariável para persistência de asma na adolescência, com base em respostas a questionários e resultados de testes cutâneos por picada. Os fenótipos clínicos foram identificados por análise de agrupamento das variáveis (*cluster*), selecionadas com base na análise de regressão logística, e comparados a respeito da persistência de asma, uso de tratamentos de controlo e avaliação funcional respiratória em idade escolar e na adolescência;

3. Estudo transversal, caso-controlo, de crianças em idade escolar, com rinite alérgica e asma, e crianças saudáveis (amostra emparelhada para a idade e género), avaliadas no que diz respeito a:

- Análise laboratorial funcional respiratória, i.e., avaliações sequenciais do débito inspiratório máximo nasal (PNIF) antes e após a aplicação de vasoconstritor tópico nasal, e espirometria com prova de broncodilatação. O teste de controlo da rinite alérgica e da asma para crianças (CARATkids) foi utilizado para a avaliação subjetiva do controlo destas doenças. As associações entre os parâmetros de avaliação funcional respiratória e de controlo subjetivo foram analisadas usando modelos de regressão linear múltipla.

- Análise laboratorial analítica por espectroscopia de ressonância magnética nuclear (NMR) para análise metabolómica não dirigida das amostras de urina e saliva de cada criança. Os dados espectroscópicos e clínicos foram analisados estatisticamente, incluindo abordagens multivariável e univariável. Adicionalmente foram colhidas amostras de condensado de ar exalado (EBC) de voluntários, em conjunto com amostras de ar ambiente da sala de colheitas. As amostras colhidas foram analisadas por NMR, com comparação dos espectros resultantes.

Resultados: A prevalência estimada de asma ativa em crianças foi de 8,4% (intervalo de confiança a 95% (95%CI) 6,6%-10,7%). As prevalências estimadas de rinite e de asma diagnosticada por médico em idosos foram 29,8% (95CI% 28,4%-31,3%) e 10,9% (95%CI 9,9%-11,9%), respetivamente. Foi encontrada uma associação forte entre asma e rinite a nível populacional, tanto nas crianças (odds-ratio (OR) 5,2, 95%CI 3,1-8,9), como nos idosos (OR variando de 8,3 95%CI 6,1-11,4 na rinite intermitente ligeira, a 39,9 95%CI 27,5-58,0 na rinite persistente moderada-grave).

No estudo de coorte, a presença de atopia e de rinite em idade pré-escolar foram fatores de risco independentes para a persistência de asma na adolescência (OR 11,8 95%CI 4,0-34,6 e OR 10,4 95%CI 3,7-29,1, respetivamente). Foram identificados três fenótipos de sibilância em idade pré-escolar, que foram preditivos para a persistência de asma, uso de medicamentos de controlo e parâmetros funcionais respiratórios em idade escolar e na adolescência. A multimorbilidade, em particular a presença de rinite, com ou sem atopia, associou-se a um pior prognóstico.

Na avaliação funcional nasal e pulmonar, observaram-se correlações entre os valores de PNIF pré e pós-vasoconstritor e do débito expiratório máximo instantâneo (PEF) e volume expiratório forçado no primeiro segundo (FEV_1), pré e pós-broncodilatação, observado independentemente da presença de rinite e asma. O melhor modelo de regressão linear múltipla para o PNIF incluiu as variáveis PEF, idade e género. Em crianças com rinite alérgica e asma, não foi encontrada associação entre o PNIF e a pontuação no questionário CARATkids, exceto no que diz respeito à obstrução nasal auto-reportada.

A análise metabolómica não dirigida em amostras de saliva e urina mostrou um subconjunto de áreas do espetro de NMR significativamente diferente nas crianças com rinite alérgica e asma, em comparação com crianças saudáveis. Alguns metabolitos que contribuíram para estas áreas do espetro foram identificados: arginina, taurina, citrato e aspartato (na saliva), e quinolinato, butirato, pantotenato, gluconato, pseudouridina e lisina (na urina). Observou-se uma correlação entre parâmetros espirométricos e a concentração urinária dos metabolitos quinolinato, butirato e pantotenato, enquanto a dos metabolitos quinolinato, gluconato e pseudouridina estava correlacionada com os níveis de óxido nítrico no ar exalado (FeNO). Observou-se uma associação entre a presença de sensibilização alergénica múltipla e as concentrações urinárias de quinolinato e salivares de citrato e aspartato. O perfil metabólico do EBC foi semelhante à composição espectral do ar ambiente.

Discussão e Conclusões: Estes estudos epidemiológicos foram os primeiros de base populacional nacional que reportaram a prevalência de sintomas de asma em todas as idades pediátricas, bem como de sintomas de rinite, sua classificação e associação com asma em idosos. Os resultados reforçaram a asma como uma doença comum em crianças e em idosos, frequentemente associada a rinite. Em crianças com sibilância recorrente em idade pré-escolar, a presença de multimorbilidade, particularmente rinite com ou sem atopia associada, tende a prever um pior prognóstico no que respeita à persistência de asma e compromisso da função respiratória em idade escolar e na adolescência. Estes resultados apoiam a necessidade de uma abordagem integrada da rinite e da asma, desde idades precoces. Para a avaliação funcional respiratória global, o PNIF pode constituir uma medida objetiva complementar à avaliação subjetiva do controlo da rinite alérgica e da asma, em crianças em idade escolar. Os resultados sugerem que na interpretação dos valores do PNIF nesta faixa etária, os valores do PEF devem idealmente ser considerados, para além da idade e do género. A análise metabolómica exploratória de amostras de urina e saliva revelou subconjuntos de áreas do espectro de NMR associadas à rinite alérgica e asma em crianças, gerando novas hipóteses que necessitam de análises suplementares. Os resultados obtidos na análise do perfil metabólico do EBC reforçaram a importância do controlo do ar ambiente durante a colheita de amostras e a necessidade de procedimentos analíticos que permitam distinguir a presença de compostos exógenos nas amostras de EBC. Em resumo, os resultados apresentados nesta dissertação adicionam evidência para uma avaliação global integrada da asma em conjunto com a rinite, tanto na prática clínica, como na investigação. Prevemos que a avaliação funcional nasal possa ser generalizada na prática clínica, numa abordagem global funcional das vias aéreas. O conjunto de metabolitos identificados na análise exploratória metabolómica estimula a continuação dos estudos nesta área para validação dos resultados, seguida da identificação das moléculas/vias metabólicas responsáveis pelas diferenças encontradas, o seu papel na fisiopatologia da rinite alérgica e asma e, por fim, como potenciais (novos) alvos terapêuticos.

Scientific Outputs

	Title	Publication status (full text)			Abstract publication
		Type of publication	Published / *in press (peer-reviewed)*	Under submission / authorship review	First author and/or presenting author
ORIGINAL CONTRIBUTIONS	*Output 1.* *Asthma-like symptoms, diagnostic tests, and asthma medication use in children and adolescents: a population-based nationwide survey*	Original article	✓ (*)		-
	Asthma prevalence in Portuguese preschool children: more scientific evidence…	Letter to the Editor	✓ (*)		
	Output 2. *Prevalence and classification of rhinitis in the elderly: a nationwide survey in Portugal*	Original article	✓ (*)		✓
	Output 3. *Prevalence of asthma and its association with rhinitis in the elderly*	Original article	✓ (*)		✓
	Output 4. *Preschool-age wheezing phenotypes and asthma persistence in adolescents*	Original article	✓ (*)		✓
	Output 5. *Lower airway patency influences peak nasal inspiratory flow in school-aged children*	Original article	✓ (*)		✓
	Output 6. *Exploratory salivary and urinary metabolomics of childhood allergic rhinitis and asthma multimorbidity*	Original article		✓	✓
	Output 7. *Room air controls in exhaled breath condensate metabolic profiling*	Letter to the Editor		✓	-
REVIEWS	*Wheezing phenotypes in childhood – is it already asthma?*	Editorial	✓		n.a.
	Non-invasive biomarkers in asthma: promises and pitfalls	Book chapter	✓		n.a.
	Metabolómica: perspectivas de aplicação na clínica pediátrica	Book chapter	✓		n.a.
	Metabolomics in asthma: where do we stand?	Review article	✓ (*)		n.a.

Legend: (*) Indexed in Medline, n.a. not applicable

Published work that is part of this dissertation (full references)

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Work under submission / authorship review

- Pité H, Morello J, Kostidis S, Verhoeven A, Morais-Almeida M, Monteiro EC, Mayboroda OA. Exploratory salivary and urinary metabolomics of childhood allergic rhinitis and asthma multimorbidity.

- Pité H, Morello J, Kostidis S, Verhoeven A, Morais-Almeida M, Mayboroda OA, Monteiro EC. Room air controls in exhaled breath condensate metabolic profiling.

Published editorial

- Morais-Almeida M, Pité H. Wheezing phenotypes in childhood – it is already asthma?. Eur Respir Pulm Dis 2015; 1:14-5.

Published book chapters / review article

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Chapter 1: Introduction

Asthma affects the lives of several hundred million people around the World, across all age groups[1]. Patients, their families and governments face high direct and indirect costs, due to healthcare resources use, loss of productivity and absenteeism of patients[2]. Asthma strongly influences the wellbeing and quality of life of patients. Death due to asthma is unbearable but still occurs. A high number of unmet needs remain to be resolved, covering many aspects of asthma related to gaps in current scientific knowledge, from epidemiology to pathophysiology and patient care.

Asthma definition: a mirror reflecting knowledge and evolution in asthma

Disease concept definition is of extreme importance. While it strikingly depends on available knowledge, disease definition is the driver of both research and clinical outcomes. Asthma is definitely not an exception.

The term “asthma” has been in use for millennia. The concept of asthma described as a medical entity exists since the time of Aretaeus about 2000 years ago[3]. However, the description of the condition, with the constellation of physical findings and signs that are currently recognized as asthma, has been in place since the XVII century[4, 5]. Asthma definition has suffered significant historical changes overtime and, importantly, asthma management progressed aligned with its concept evolution (Figure 1).

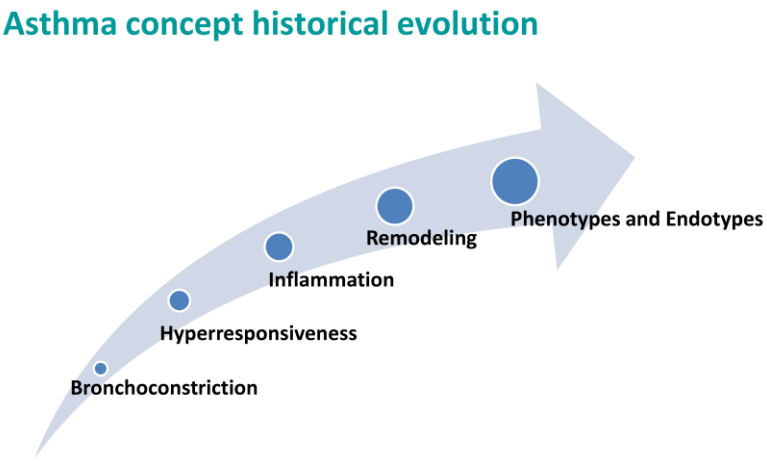


Figure 1 – The asthma concept historical evolution

The original concept of asthma as a primary disease of airways smooth muscle drove the development of bronchodilator drugs, particularly since the beginning of the last century[5]. The mainstay of asthma treatment was the relief of bronchospasm and its accompanying signs and symptoms of cough, wheezing, chest tightness, dyspnea and fatigue. The concept of airway hyperresponsiveness, a hallmark of asthma, came later. It is defined as an exaggerated response of the airways to various stimuli resulting in airways obstruction. However, the main step in asthma evolution occurred with the characterization of asthma as an inflammatory disease. This concept changed the view of asthma from an episodic disease of bronchoconstriction into a chronic disease of inflammation, from bronchodilators rescue treatment to corticosteroids preventive treatment. Corticosteroids were not generally available for medicinal use until the late 1940s. In the early 1950s, anecdotal reports of refractory asthma patients that had a significant response to systemic corticosteroids were published[4]. By the 1970s, systemic corticosteroids had been

accepted as the standard therapy both to treat and to prevent asthma exacerbations, and the major issues were how to avoid these treatments' severe side effects for patients requiring long-term medication use. The solution to this problem came in the form of inhaled corticosteroids. These were slowly accepted and introduced into clinical practice over the next decades and are nowadays the first line preventive treatment of asthma in all age groups[6, 7]. This change in the paradigm of asthma and its management was associated with the falling asthma mortality and disability rates over the last decades[1, 8-10].

Asthma is not only characterized by bronchoconstriction and inflammation but also by structural changes in the airways, generally known as airway remodeling. These features include epithelial mucous metaplasia, increased thickness of the subepithelial *lamina reticularis*, deposition of matrix proteoglycans and collagen in the submucosa and between bundles of smooth muscle, increase in smooth muscle and proliferation of microvessels[5]. Airway remodeling is not fully dependent on inflammation and may be present from the pre-symptomatic inception of the disease[11-13]. The acknowledgment of the possibility of fixed or irreversible airflow limitation in some patients with asthma, recognized as an adverse asthma outcome, was another relevant step in the asthma concept evolution[6].

In the last years, another significant change in the asthma definition took place. One main historical reason for some of the current existing gaps on asthma pathophysiologic mechanisms knowledge may have relied on disregarding its complexity and defining asthma as a single disease entity. Although the heterogeneity of asthma has been recognized for over a century, for instance as "intrinsic" and "extrinsic" asthma[14], it was only in 2014 that the Global Initiative for Asthma (GINA) published a new asthma syndrome definition: asthma was no longer considered a chronic inflammatory disease of the airways but rather a heterogeneous disease, usually characterized by chronic airway inflammation[6]. Nevertheless, the diagnosis of asthma should still be based on a history of characteristic symptoms patterns and evidence of variable airflow limitation from bronchodilator reversibility or other tests[6].

Current asthma definition[6]

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.

This more recent change of concept is again driving current research developments into the search for asthma phenotypes, i.e., groups of patients that share similar observable characteristics. Most importantly, current efforts are to look for asthma endotypes, defined as subtypes of asthma which may be clinically overlapping, but each being caused by distinct underlying pathophysiological mechanisms, with different treatment and prognosis[15]. This may actually lead to different diseases definitions, perhaps making the current concept of asthma obsolete in the future. The need for a new approach to the classification and management of airway disease through the identification of key causal mechanisms and treatable traits has been advocated[16, 17].

Optimal asthma control is the primary current goal of asthma management

With no established curative treatment, national and international guidelines for asthma management have identified that the primary goal of management is to achieve asthma control[6, 7]. This consists of two domains: symptoms control and reduced future risk of adverse outcomes. These two domains are not independent (e.g., well-controlled asthma symptoms significantly reduce future risk of adverse outcomes).

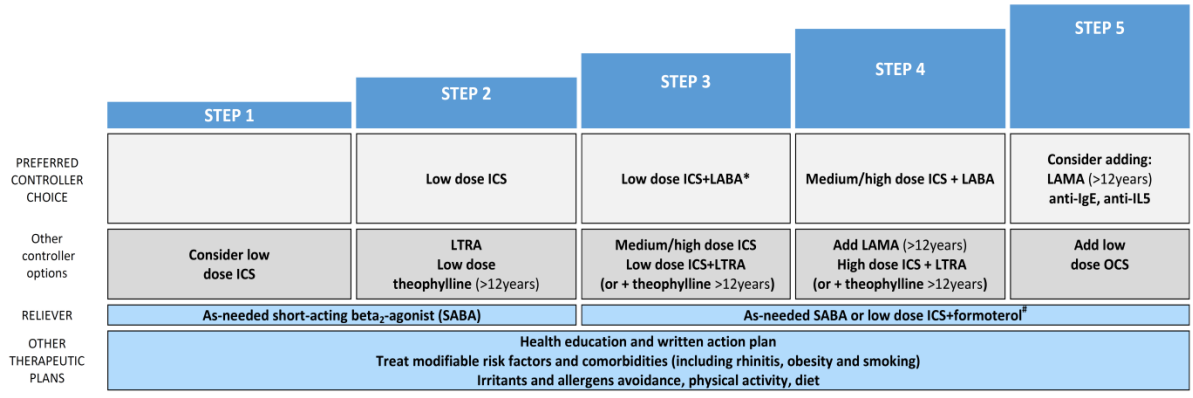
Asthma symptoms control is classified into three categories (well-controlled, partly controlled and uncontrolled), according to daytime and night-time symptoms, activity limitation due to asthma and need of reliever therapy (Table 1)[6, 7].

Table 1 – Asthma symptoms control levels (adapted from[6, 7])

Symptoms control level	Well-controlled	Partly controlled	Uncontrolled
In the past 4 weeks: Daytime asthma symptoms in ≥2 days/week? Any night waking due to asthma or cough? Reliever needed for symptoms ≥2 days/week? Any activity limitation due to asthma? (adapted to age group)	None of these	1 to 2 of these	3 to 4 of these

n.b., any exacerbation in any week identifies uncontrolled asthma and demands for re-assessment of the preventive treatment plan.

Reducing future risk includes three main aspects: minimizing asthma exacerbations, long-term lung function impairment and medication side-effects. Asthma management should be adapted to every patient in order to maintain asthma control, without exacerbations and with the best possible lung function, using the minimum needed medication. Given asthma symptoms characteristic variability, asthma management requires regular control assessment, in a close partnership between patients and physicians and other healthcare professionals, empowering patients to actively achieve total disease control as a goal. Pharmacological and non-pharmacological strategies should be addressed and combined in educational plans for asthma management. Prevention and avoidance of risk factors that may precipitate asthma symptoms are key elements, as well as multimorbidity control, together with stepwise pharmacotherapy (Figure 2).



* For children 6-11 years, the preferred choice is medium dose ICS; # low dose budesonide or beclomethasone associated with formoterol used both for maintenance and reliever treatment regime for adolescents and adults.

Legend: ICS – inhaled corticosteroid, FEV_1 – forced expiratory volume in one second, IgE – immunoglobulin E, IL-5 – interleukin 5, LABA – long-acting beta-agonist, LAMA – long-acting muscarinic antagonist (tiotropium), LTRA – leukotriene receptor antagonist, OCS – oral corticosteroid, SABA – short-acting beta-agonist.

Figure 2 – Stepwise asthma management (adapted from [6, 7])

In selected cases, the new GINA guidelines considers the use of sublingual allergen specific immunotherapy for adults[6]. Current evidence also supports this treatment by subcutaneous route and its use in children, but responses can be specific to extracts and treatment regimens[6, 18, 19]. Studies using validated tools to assess disease control, standardized outcomes (such as exacerbations) and comparing immunotherapy with other pharmacological therapy should bring additional relevant evidence to this field.

The global airways disease context

The need to consider asthma multimorbidity is critical to achieve asthma control. The nose strategic position at the entry of the airways justifies its crucial role in lower airways homeostasis. Upper and lower airways are a continuum, linked not only anatomically but also through neural reflexes and systemic pathways (Figure 3)[20-22].

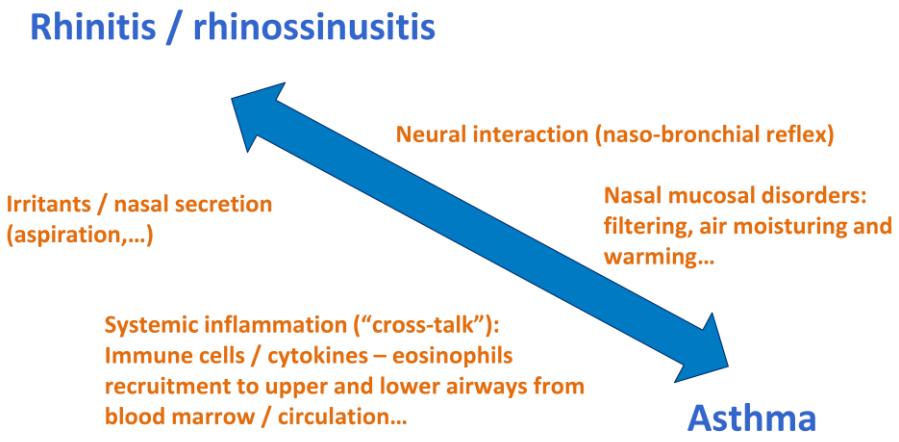


Figure 3 – Nose-lung interaction mechanisms[21, 23]

Rhinitis is the leading comorbidity in asthma; most patients with asthma have rhinitis and up to 40% of patients with rhinitis have asthma[6, 22]. Chronic rhinosinusitis with/without nasal polyps is also associated with asthma[24]. Both rhinitis and rhinosinusitis clearly influence asthma control[6, 22]. Failure to consider the treatment of coexisting rhinitis as essential to the management of asthma may impair clinical control of the latter and has been associated with high frequency of asthma-related hospitalization and emergency department visits[6, 21, 23]. The close relationship between asthma and rhinitis has been recognized by the World Health Organization, namely through the Allergic Rhinitis and its Impact on Asthma (ARIA), and catalyzed by the Global Alliance against Chronic Respiratory Diseases (GARD) initiatives[22, 25]. Current available evidence mostly regarding adults supports that patients presenting with chronic airway symptoms should be specifically and actively asked about upper and lower respiratory symptoms, and these need to be addressed in unified therapeutic plans.

Although the interaction between chronic upper and lower airway inflammation has primarily been studied in allergic individuals, not only allergic but also infectious or non-allergic non-infectious rhinitis can be involved (Figure 4). Currently, rhinitis is defined as inflammation of the mucous membrane of the nose and is characterized by classical symptoms of nasal pruritus, sternutation, rhinorrhea and/or nasal obstruction[22, 23]. At least two nasal symptoms should be present during two or more consecutive days for more than one hour on most days[23].

Infectious rhinitis is often an acute and self-limiting disease caused by a virus, usually known as common cold. However, infectious rhinitis may have a more prolonged disease course; discolored secretions and/or crust formation are clinical landmarks of this rhinitis subtype[26].

Allergic rhinitis is considered when nasal symptoms are elicited by immune mediated mechanisms after allergen exposure, such as of house dust mites, pollens, molds or pets. Systemic atopy is usually investigated by skin prick tests or serum specific immunoglobulin (IgE) against allergens. Local allergic rhinitis has also been identified, as the occurrence of allergic rhinitis symptoms in patients with exclusively local production of allergen specific IgE antibodies in the nasal mucosa[26, 27].

Non-allergic non-infectious rhinitis involves a heterogeneous group of patients suffering of rhinitis without clinical signs of infection and allergic inflammation. In reality, more than one etiologic factor may coexist in the so-called mixed rhinitis[26].

Asthma has been associated with allergic and non-allergic rhinitis subtypes but a distinct endotype of asthma associated with allergic rhinitis has been suggested[26, 28, 29].

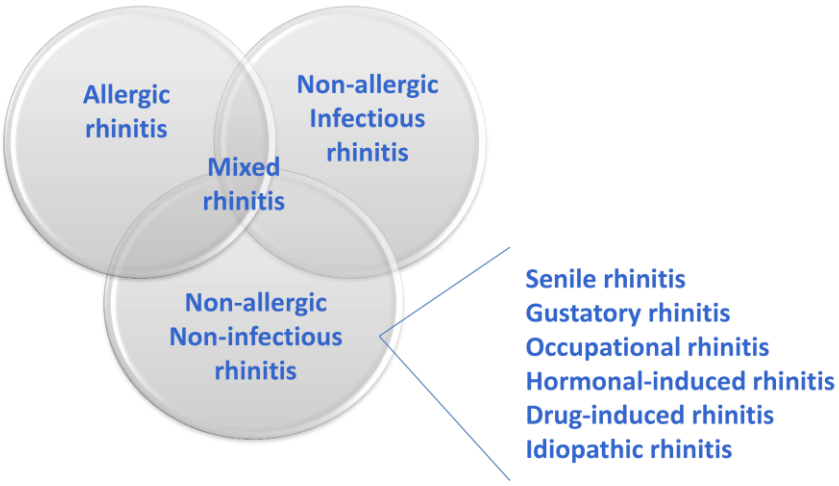


Figure 4 – Classification of rhinitis (adapted from [26])

According to the ARIA guidelines, allergic rhinitis should be classified for its duration and severity (Table 2)[22].

Table 2 – Allergic rhinitis definition and classification (adapted from [22, 23])

Allergic rhinitis definition Inflammation of the mucous membrane of the nose, characterized by nasal pruritus, sneezing, rhinorrhea and/or nasal obstruction, elicited by immune mediated mechanisms after allergen exposure.	
Allergic rhinitis classification	
Duration	**Nasal symptoms duration**
Intermittent	Lasting <4 days/week or <4 consecutive weeks
Persistent	Lasting for at least 4 days in a week and for >4 consecutive weeks
Severity	**Sleep disturbance, impairment of daily activities, leisure and/or sports, impairment of school or work, and troublesome symptoms**
Mild	None of the above
Moderate-Severe	At least one of the above is present

Allergic rhinitis pharmacologic treatment is based on antihistamines in all rhinitis classification categories, and topical nasal corticosteroids are the mainstay of rhinitis control in persistent rhinitis and in all moderate-severe presentations[22]. Allergens and irritants avoidance, nasal hygiene and correct use of topical nasal drugs are essential measures for successful rhinitis control. If accurately prescribed and monitored in selected cases, allergen specific immunotherapy may allow prolonged symptoms remission and reduce the need for medication: through its immunomodulatory effect it may modify the natural history of the allergic disease, decrease the emergence of new allergic sensitizations, as well as the incidence of asthma in children with allergic rhinitis[23, 30-33]. Other pharmacologic treatments in allergic rhinitis include vasoconstrictor drugs (for short-term periods, useful for the relief of nasal obstruction) and leukotriene receptor antagonists (anti-inflammatory optional controller therapy)[22].

Rhinitis might not appear to be serious *per se* because, without complications or associated diseases, it is not linked to severe life-threatening morbidity. However, rhinitis is regarded as a public health problem; its prevalence, burden and costs are substantial[22, 34-36], even without adding all possible complications of the disease and commonly associated conditions, such as asthma.

In spite of the advances with the development and dissemination of guidelines for the management of asthma and associated diseases[6, 7, 22], national and international surveys continue to reveal inadequate control, as is the case in Portugal[37-44]. Asthma and its multimorbidity continue to impart a significant onus on individuals with the disease, families of the affected and society and health economies. Identifying current unmet needs and establishing strategies for their resolution are important to contribute to the reduction of these diseases burden.

Unmet need: asthma prevalence and its association with rhinitis in the extremes of life

Despite the fact that asthma has been recognized since more than 2000 years ago, it was only in the last four decades that asthma has become a serious health concern. This was precipitated by an epidemic of asthma deaths in the late 70s, which occurred in different places, mainly New Zealand, Australia, The United Kingdom, Canada and the United States of America. With improved asthma patients care, mortality rates have fallen worldwide over the last three decades, but asthma remains a major cause of chronic disability, affecting all generations[1, 8-10].

Chronic respiratory diseases, including asthma and rhinitis, represent a global concern. The World Health Organization recommends assessing population needs to better define adequate health policies[45]. Epidemiologic studies at the population level have been crucial for this assessment. Over the last decades, large international surveys have been conducted using standardized methodology to estimate asthma symptoms prevalence, including the European Community Respiratory Health Survey (ECRHS), the International Study of Asthma and Allergy in Childhood (ISAAC) and the Global Allergy and Asthma Network of Excellence (GA^2LEN) surveys[46-49]. While the ISAAC study focused on children (aged 6-7, 9-11 and 13-14 years)[47, 48], the ECRHS assessed the prevalence of asthma symptoms in the general population aged 20 to 44 years[46]. The ECRHS was conducted in different countries (mostly in Western Europe) for the first time in 1991-1994. The GA^2LEN survey included a broader age group (15-74 years) in 15 European countries in 2008/2009[49]. All these studies have found wide variations in the prevalence of asthma symptoms in the different countries: more than 5% of any investigated population suffered from asthma; in some regions, this percentage was much higher. The reasons for the variability in asthma frequency across the world and the sharp rise in its prevalence in the last decades have been mostly associated with environmental factors and lifestyle, which have undergone profound changes in a relatively short period of time (including changes in housing design, exposure to outdoor and indoor pollutants, microbial exposure, family size and childcare arrangements, diet and sedentary life style). However, the effect of specific environmental exposures can be different amongst individuals with different genetic predispositions or be modified by co-exposures, and knowledge about the underlying cause(s) of asthma epidemics remains elusive.

Portugal participated in the three surveys mentioned before[46, 49, 50], but only some cities were included and the results were limited to the predefined age groups. Additionally, nationwide health surveys took place[51, 52], but their focus had not been asthma and therefore a standardized methodology to provide data on asthma symptoms wasn't used. The Portuguese National Asthma Survey – Inquérito Nacional sobre Asma (INAsma) was the first population-based nationwide epidemiological study that estimated asthma prevalence in Portugal in all age groups[53]. It was conducted in 2010 and included a large representative sample of the Portuguese population. The estimated prevalence of lifetime asthma (defined as positive answer to the question "*Have you ever had asthma?"*) and current asthma (defined as self-reported lifetime asthma and "*wheezing*", "*waking with breathlessness*" or "*having an asthma attack*" in the last 12 months) were 10.5% (95%CI 9.5%-11.6%) and 6.8% (95%CI 6.0%-7.7%), respectively[53]. This study further showed a strong association of asthma with rhinitis and rhinosinusitis, in agreement with the known risk of chronic disease of upper airways in asthmatics[53]. These data were important to provide accurate estimates of asthma at the national level. Still, two particularly vulnerable and internationally data-lacking populations remained on the extremes of life: the children and the elderly.

Differences in symptoms and in asthma and rhinitis frequencies could be expected in these age groups, possibly due to age-related physiological changes including structural and functional airway changes, immune system development/senescence, or different lifetime environmental exposures. The concept of the "allergic march" described the progression from atopic dermatitis in infancy to subsequent asthma and allergic rhinitis in later childhood and adolescence[54], which could suggest age-related changes in the

frequencies of these diseases. Comorbidities and medication intake could also account for some differences in these two population groups. A better understanding of the asthma and rhinitis impact within specific childhood and elderly age groups is important to reduce the diseases burden in these population groups.

The ISAAC study had changed our knowledge about worldwide prevalence of asthma-related symptoms in children. Portugal had been involved since this project's early beginning in 1991 and participated in all study phases: phase I in 1994/1995; phase II in 2000/2001 and phase III in 2002. However, again, neither ISAAC, nor other previous studies performed in Portugal estimated the prevalence of asthma across all pediatric ages (<18 years) or among a representative nationwide sample. Internationally, nationwide studies on the prevalence of asthma across the entire pediatric population had only been reported in Germany and the United States of America[55, 56], and no study had reported the prevalence of asthma-like symptoms, other than wheezing, across all pediatric ages.

On the other extreme of life, elderly-targeted, population-based studies considering asthma and rhinitis were scarce, although the greatest burden of asthma deaths occurred among elderly subjects[10, 56-58]. In recent decades, there was a significant increase in the elderly population, both in Europe and the United States of America. Studies specifically addressing this age group were needed for a proper analysis of asthma and its association with rhinitis in geriatrics, allowing better knowledge about this population subgroup needs and raising awareness towards effective disease control also in this age group. Nationwide epidemiologic data on rhinitis in the elderly did not exist before at the international level and no population-based study had ever analyzed the association between asthma and rhinitis symptoms and severity in this age group.

Unmet need: definition of early childhood wheezing phenotypes related to asthma persistence

Knowledge on the frequency of the disease at the population level, the characterization of its symptoms and its multimorbidity is important to increase knowledge on the disease and to better plan strategies aiming to reduce its burden. Yet, in order to achieve this aim, an early recognition of the diagnosis is essential.

Most asthma cases begin during childhood. Wheezing in early childhood is among the most frequent respiratory symptoms[59-61]. When evaluating a child with wheezing, it is important to exclude other causes than asthma, from aspiration to respiratory infections or other pulmonary, cardiac or even gastroesophageal reflux diseases (Table 3).

After excluding these disorders, much heterogeneity remains in recurrent wheezing, which may be associated with very distinct prognosis. Most children with early wheezing become symptom-free in later childhood and adolescence; wheezing is transitory and the children fully recover without sequels, having excellent prognosis. On the other hand, early-onset wheezing is also associated with persistent asthma symptoms in adulthood and, with more severe, persistent lung function impairment[62-64]. Thus, distinguishing early wheeze phenotypes to predict long-term asthma persistence is of major clinical relevance. The recognition of such distinct outcome groups is valuable for informed counselling to parents and a prerequisite for phenotype-specific management, identifying early those children who require closer follow-up and active treatment, from those with good prognosis and in need for minimum intervention.

Table 3 – Differential diagnosis of asthma in children

Differential diagnosis (non-exhaustive list of diseases that may manifest with wheezing other than asthma)
Respiratory infection, including bronchiolitis, bronchitis, laringotracheobronchitis
Aspiration of foreign body, associated with neuromuscular disorders, fistulae (e.g. tracheoesophageal)
Gastroesophageal reflux disease
Vocal cord dysfunction
Tracheobronchomalacia
Cystic fibrosis
Immune deficiency
Primary ciliary dyskinesia
Bronchopulmonary dysplasia
Bronchiolitis obliterans
Pulmonary edema
Interstitial lung disease
Cardiovascular disease, including left-right shunt, heart failure, vascular ring
Mediastinal mass, including aneurysm, tumor, goiter
Characteristics that favor differential diagnosis
Sudden onset
Early age of symptoms onset
Context: exposure to infectious agents, irritants, relation to food/drink intake
Systemic signs and symptoms: weight loss, failure to thrive, asthenia, anorexia, night sweats
Recurrent / severe infections
Hemoptysis
Atypical disease progression: persistent signs and symptoms; symptoms not related to common triggers such as exercise, allergen exposure, stress, cold; inadequate therapeutic response
Asymmetric signs
Lack of personal or family history of rhinitis, atopic dermatitis, food allergy or family history of asthma

The largest contributions to identify early childhood wheeze phenotypes came from longitudinal birth-cohorts[60, 62, 64-67], and from mostly moderate to severe wheezing cohorts studies[68-70]. However, different studies originated distinct phenotypic classifications. Many categories of recurrent wheezing were based on predefined hypotheses and limited to single disease dimensions. Therefore, its application to different age groups or incorporation of other characteristics was restrained. Furthermore, phenotype classifications based on temporal criteria have limited clinical use, since such groups can only be established retrospectively. In 2008, a European Respiratory Society Task Force recommended distinguishing between two wheezing phenotypes with a differentiated approach to preventive treatment: episodic viral wheeze and multiple-trigger wheeze[71]. However, this classification has been revised due to difficulties in including children in mutually exclusive groups and to the rapid symptom pattern change over time[72]. The international consensus group review acknowledged that this classification of wheeze was a relatively poor predictor of long-term outcome[72].

In more recent years, multivariable statistical methods have been proposed to facilitate the unbiased identification of relevant phenotypes[73]. Such groups may not be directly observable and must be determined from objective data. Statistical methods designed to detect clusters underlying multivariable

data have the advantage of avoiding the need to define phenotypes by the onset of wheeze at a given age or other pre-specified criteria, and thus are classified as “hypothesis-free” methods. Another benefit is to simultaneously consider several disease dimensions. Since there is no specific single indicator that accurately predicts the development of asthma or identifies high risk children or the disease course of an asthmatic patient, it is most likely that the combination of multidimensional features will be necessary to achieve these aims. These methods tend to have a broader application, better allowing comparisons in different population settings. Application of such “unbiased” statistical methods “without predefined hypothesis” to distinguish groups of patients is important to validate previous phenotypic classifications.

In brief, “unbiased” phenotypic classifications derived exclusively from data can be complementary to groups defined *a priori* or based on directly observable criteria. The identification and characterization of distinct wheezing phenotypes with different prognosis, by the comprehensive analysis of longitudinal datasets are important to implement interventional measures to reduce the asthma burden since pediatric age (author publication – full text available in the Attachments sections – number 1)[74].

Unmet need: biomarkers in allergic rhinitis and asthma multimorbidity in children

According to the current paradigm, asthma should not be regarded as a single disease, but rather a syndrome of complex, multiple, overlapping “subtypes”, which are probably distinct since their early beginning. The asthma syndrome is multifactorial, with genetic, lifestyle and environmental components interaction. Under the umbrella of current asthma definition, age of onset of symptoms, associated diseases, symptoms presentation and its triggers, lung function impairment, inflammation patterns and airway remodeling features can differ and be used to define different phenotypes. The prognosis is also distinct as well as the response to therapy. Despite all efforts to define specific asthma phenotypes, no single consensual phenotypic classification of asthma exists as, to date, no strong relationship has been found between specific pathological features and particular clinical patterns or treatment responses[6]. Nevertheless, “allergic asthma” (as defined by GINA) is the most commonly recognized phenotype[6].

“Allergic asthma” is characterized by eosinophilic airway inflammation and airway hyperreactivity driven by adaptive T helper (Th) 2 cells that are stimulated by dendritic cells, under the influence of epithelial cytokines interleukin (IL)-33, IL-25 and thymic stromal lymphopoietin, to produce IL-5, IL-13 and IL-4[75]. The latter drives IgE synthesis, which has the potential to activate mast cells and basophils. Mast cells can infiltrate the airway, contributing to bronchial hyperreactivity and also plasma extravasation. Moreover, the presence of IgE on dendritic cells primes naive T cells for Th2 differentiation and lowers the atopic individual’s threshold to mount allergen-specific T cell responses. Through the production of IL-5, Th2 cells drive eosinophils development, survival and activation. Upon activation, eosinophils undergo cytolysis and release extracellular deoxyribonucleic acid traps that can lead to high local concentrations of eosinophilic toxins that can damage structural cells of the lung[76]. These traps can also contribute to the thick mucus, with the characteristic Charcot-Leyden crystals. Via IL-13, Th2 cells can also cause goblet cell metaplasia producing mucus, airway hyperreactivity and extravasation of inflammatory cells. Interleukin-13 activated epithelial cells produce angiogenic growth factors and periostin that promotes myofibroblast proliferation[77]. The disrupted epithelial barrier also contributes to remodeling through epidermal growth factor driving the production of transforming growth factor beta and consequent deposition of collagens, laminin, tenascin and proteoglycans. Eosinophils are also an important source of transforming growth factor beta. Besides this long considered Th2 disease, it is now known that some asthmatic inflammation is neutrophilic, with predominant mixed Th1 and Th17 cytokine milieu, and that some eosinophilic inflammation is rather controlled by type 2 innate lymphoid cells producing IL-5 and IL-13 in response to other common triggers such as pollutants and viruses. In all asthma cases, mixed

populations of different cell types and cytokines are found in the airways and a dynamic overlap of these mechanisms is probably occurring[75].

The predominant "allergic asthma" phenotype typically begins during childhood and is associated with other allergic diseases, mainly allergic rhinitis[6]. This well-known link between asthma and allergic rhinitis has recently been supported by large-scale unbiased clustering at the population level in children, suggesting that undisclosed mechanisms underlying these diseases need to be investigated considering an "allergic multimorbidity" cluster[28, 29]. At the clinical level, this further supports an integration of care pathways in these allergy-related diseases.

Integrating care pathways with biomarkers – adding nasal function in concurrent evaluation of allergic rhinitis and asthma control in children?

The recognition of the close relationship between asthma and rhinitis at the global level has resulted in a call for a change in patients care, with the combined approach for asthma and its multimorbidity, particularly allergic rhinitis. In this context, the need for a simple assessment tool that could be readily used in clinical practice has been overcome with the development of the Control of Allergic Rhinitis and Asthma Test (CARAT)[78-80]. This questionnaire, designed by a group of Portuguese immunoallergologists, respiratory physicians, family physicians and pediatricians, was the first to assess the control of both diseases concurrently. It has been validated and adopted in different languages[81]. Furthermore, website and smartphone applications have been developed and a free open model distribution was adopted. A pediatric version of this test – the CARATkids – has also been developed and validated[82, 83]. The use of validated subjective scoring tools is nowadays highly recommended for the subjective evaluation of rhinitis and asthma control[6, 22, 84, 85].

Adding an objective measurement to subjective scores can be useful to improve disease control. One of the main complaints in allergic rhinitis is nasal obstruction[23, 85, 86]. It has been previously shown that subjective and objective measures of nasal patency do not correlate well[87]. Thus, it is recommended that the assessment of a patient suffering of nasal obstruction should be based upon both measures[84, 85, 88]. However, no previous data existed on the association between CARAT questionnaires scores and objective measures of nasal patency.

Nasal airflow can be objectively and easily assessed using a nasal peak flow meter. Peak nasal inspiratory flow (PNIF) is the simplest validated measure of nasal function[84, 85], which has been successfully used for the objective evaluation of rhinitis and its control[89-92], including in children[93, 94]. Reference values for the pediatric population have been published, but no data was previously available on PNIF values after decongestion[95-99]. It is important to measure PNIF values before and after decongestion to elicit the role of mucosal swelling[84, 85].

The impact of lower airway patency on PNIF also needs to be considered[84, 85, 100-102], especially when addressing patients with the rhinitis and asthma multimorbidity phenotype. This had seldom been analyzed in pediatric age. A bivariate correlation between baseline PNIF and peak expiratory flow (PEF) had been described in healthy children[98]. Nevertheless, since a continuous increase in PNIF values has been consistently reported with children's age[95-99], it is important to exclude that the observed association between PNIF and PEF is not a simple reflection of the normal growth (i.e., older children have larger airways and correspondingly higher nasal airflow). Most studies in children also describe an association between PNIF and height, weight and gender[96-98]. Analyzing the association between PNIF and lower airway patency in children using multivariable models is needed for a more accurate use of nasal airflow assessment in this age group, possibly contributing with additional information regarding rhinitis and asthma concurrent control evaluation.

"Unbiased" molecular profiling – contributing to the link between clinical phenotypes and disease endotypes?

The diversity of asthma phenotypes led to the hypothesis of existing distinct undisclosed pathophysiological mechanisms, but presently no agreed criteria upon asthma endotypes exists. Exploratory molecular profiling using high-throughput "omics" approaches may support the "unbiased" characterization of clinically defined asthma phenotypes at the molecular level and thus help to step forward into defining asthma endotypes (Figure 5)[103].

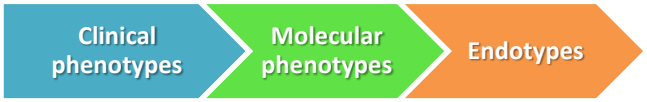


Figure 5 – Progression from phenotypes to endotypes[103]

Presently, asthma pharmacologic treatment continues to be based on the intrinsic asthma severity, with drugs added on the basis of disease control, approximate to a "one size fits all" regime. Although this approach benefits many patients, its efficacy varies widely at the individual level. Asthma management is still mostly "reactive" (added treatments after loss of asthma control) rather than "proactive" (acting earlier to prevent disease burden), although more emphasis is now given to future risk reduction[6, 7]. In this setting, biomarkers are needed to innovate asthma management. Currently available and most promising biomarkers have been discussed elsewhere (author publication – full text available in the Attachments section – number 2)[104].

Uncovering the mechanisms underlying different endotypes in the "asthma syndrome" would improve knowledge on the natural history of these conditions and ideally lead to mechanism-specific pathophysiologic biomarkers and interventions for disease prevention and treatment with effective personalized therapies.

The complexity of asthma can rely on several pathophysiological mechanisms, which can interact and may not be present in all patients or at all times. Multivariable measurements are most likely needed to capture asthma complexity, which may yield more useful information than existing single or even panels of combined biomarkers[104]. In this context, the "omics" disciplines, combining the high-throughput analytic techniques with bioinformatics, have recently emerged as important tools in medical research. These provide comprehensive, broad molecular analysis in biological specimens and may enable the detection of multivariable composite biomarkers of the disease. Among the "omics" disciplines, metabolomics is the closest to phenotype expression, aiming at the universal analysis of metabolites in biological specimens (Table 4; author publications – full texts available in the Attachments section – numbers 3 and 4)[105, 106].

Table 4 – Metabolomics and related concepts definitions[106]

Concept	Definition/characterization
Metabolomics	Large-scale study of metabolomes that aims at the universal analysis of low molecular weight (typically <1500Da) metabolites in biological specimens.
Metabolome	Complement of naturally-occurring and exogenous metabolites within biological systems.
Metabolite	Substrate and product of metabolism that drive essential cellular functions, such as energy production and storage, signal transducing and apoptosis.
Metabolism	Chemical reactions that occur within an organism to sustain life. The reactions are globally divided into two categories: catabolism (the breakdown of organic matter to produce energy) and anabolism (the construction of molecules from smaller units).
Targeted metabolomics	Metabolomics methods developed and optimized to measure the concentrations of a predefined set of metabolites of interest. These methods allow higher sensitivity and selectivity and may provide analytical validation to results from untargeted analysis.
Untargeted metabolomics	Metabolomics methods aimed at measuring the broadest range of metabolites present in an extracted sample without *a priori* knowledge of the metabolome.
Mass Spectrometry	One of the main analytical platform used for metabolomics analysis that measures the masses of molecules and their fragments (mass-to-charge ratio (m/z) of ions that are formed by inducing the loss or gain of a charge from neutral species) to determine their identity. Usually, proceeded by a separation approach (e.g., liquid or gas-chromatography).
Nuclear Magnetic Resonance	One of the principal analytical techniques used for metabolomics analysis that exploits the magnetic properties of the atom nuclei (absorption and re-emission of electromagnetic radiation) to obtain physical, chemical, electronic and structural information about molecules.

In contrast to transcriptional, translational and post-translational changes, metabolites have the distinct advantage of being more proximal markers of disease processes and to easily capture the effect of past exposures. The profiling of metabolites in biofluids, cells and tissues allows an instantaneous snapshot of a biological system status, reflecting the net results of genetic and environmental interactions, which are two critical dimensions in asthma.

Classically, the molecular characterization of asthma has been driven by hypotheses generated by investigators. This strategy relies on a specific number of analytes and previously set criteria to classify the disease. The complexity of pathophysiological processes underlying asthma rendered a broad spectrum of involved metabolic pathways and products, resulting in an overwhelming choice of potential biomarkers and targets for disease treatment[104]. This classical approach for the molecular characterization of asthma may be further complemented with a different strategy. For instance, untargeted metabolomics has no metabolite identification before sample analysis and provides a broad and unsupervised multivariable description of metabolites in a given sample (Table 4). As such, it may contribute to “unbiased”, data-driven metabolic profiling of asthma. The utility of such characterization can be to identify potential biomarkers for asthma or asthma “subtypes” and to improve understanding of the pathophysiology of asthma, with a “hypothesis-free” strategy[107].

Mass spectrometry and NMR are the two principal analytical platforms used for metabolomics analysis (Table 4). These two techniques are complementary and different molecules can be identified. Furthermore, the NMR is highly reproducible and usually requires less sample preparation but has lower sensitivity, while liquid or gas chromatography coupled with mass spectrometry is highly sensitive and specific but depends on sample preparation that may affect the results[106].

The metabolome also highly depends on the sample source (Table 5).

Table 5 – Pros and Cons of main biomarker sample sources in asthma metabolic profiling studies[104]

Biomarker source	*Pros*	*Cons*
Lung biopsy / bronchoalveolar lavage fluid	More direct airway evaluation Molecular, cellular and tissue biomarkers	Invasive Several medical contraindications Rescue medication / procedures needed Non-repeatable in many patients Expertise and experience required for procedure Procedure itself may induce changes in airways
Induced-sputum	More direct airway evaluation Semi-invasive Molecular and cellular biomarkers	Very difficult in young children Contraindicated in severe bronchoconstriction / active cardiovascular disorders Rescue medication / procedures needed Non-repeatable over short time-period (<12-18h) Procedure itself may induce changes in airways Require expertise, expensive and time-consuming specialized lab assays
Exhaled breath	More direct airway evaluation Totally non-invasive Point of care technology May be collected across all ages May be collected in severe patients Allows serial measurements	Highly variable matrix Highly diluted soluble markers (very low concentrations)
Blood	Minimally-invasive Molecular and cellular biomarkers May be collected across all ages May be collected in severe patients Allows serial measurements	Not directly reflecting the airways
Urine / Saliva	Totally non-invasive May be collected across all ages May be collected in severe patients Allows serial measurements	Not directly reflecting the airways

Metabolomics applied to lung tissue may hold the best chance of obtaining relevant data for detecting specific asthma endotypes, given the direct airways evaluation. However, obtaining lung tissue requires invasive procedures that are difficult to set up. Biomarkers need to be developed in accessible compartments that can be analyzed relatively easy and repeatedly. More indirect samples such as blood or even urine samples have been shown to be useful for metabolic profiling biomarker identification and even underlying mechanisms research in asthma[105-108]. These preliminary metabolomics data reinforced these matrixes as surrogates of pathophysiologic processes that occur in asthma. Urine is particularly easily obtained non-invasively and it is metabolically interesting as an end-product sampling of metabolic activity of a given organism[109]. Recently, liquid chromatography combined with mass spectrometry was applied to a pilot study of saliva samples in asthmatics, also demonstrating the potential to discriminate asthma[110]. Exhaled breath analysis is also very appealing, given the easy and repeatable sampling that cope with asthma dynamic changes. In fact, exhaled breath can be sampled in a gaseous (vapor) or liquid (exhaled breath condensate - EBC) state, in fully non-invasive ways across all age groups. Exhaled breath potentially reflects the airways[104]. However, when measuring exhaled molecules as biomarkers of physiological processes, one should consider that many compounds in exhaled breath may have exogenous origins. So far, the search for useful molecular biomarkers in this matrix has been hampered by methodologic difficulties mainly dealing with samples stability, very low molecular concentrations, low sample volumes, variability and lack of sampling and analyzing methods standardization[104, 111]. EBC has the advantage of being a more stable matrix than exhaled breath vapor, including volatile and also non-

volatile compounds. It is obtained by cooling exhaled air and is thought to reflect the composition of the airway lining fluid. Although methodological recommendations for exhaled breath sampling and analysis have been published[112, 113], the procedures for exhaled breath collection and biomarker detection are not fully systematized and there is significant heterogeneity between different working groups, hampering results comparisons[112, 113]. This heterogeneity in procedures becomes even more important with the growing number of "omics" studies using such multivariable analytical techniques as NMR and mass spectrometry. Addressing these issues of procedures standardization, together with metabolite identification and external validation will be critical in order to achieve robust conclusions that can be translated into clinical practice, using these high-throughput technologies at their full potential[105, 106]. In addition to biomarkers identification, relating metabolites to their biological role may bring relevant data for understanding the biology underpinning asthma heterogeneity and ultimately for specific targeted therapies[106].

Although metabolomics in asthma is at its early infancy, novel biological insights have already been gained[105, 106]. Currently, several clinical studies using untargeted metabolomics approaches have yielded distinct results and suggested a broad number of metabolites associated with asthma. Common altered individual metabolites identified by different research groups include amino acids, lipids, purines, salts and alcohols (Table 6).

Table 6 – Metabolites associated with asthma in at least two independent studies in humans comparing untargeted metabolomics profiles of asthmatics with non-asthmatics

Identified metabolite	Class*	Metabolomics analysis	Samples	Participants	References
Adenosine	Purine	MS[114-116], NMR[117]	EBC[114, 117], Plasma[115], Serum[116]	Children[114], Adults[115-117]	(114-117)
Arginine	Carboxylic acid (amino acid)	MS[116], NMR[117, 118]	EBC[117], Serum[116, 118]	Adults[116-118]	(116-118)
Phenylalanine	Carboxylic acid (amino acid)	MS[116, 119], NMR[117]	EBC[117], Serum[116, 119]	Adults[116, 117, 119]	(116, 117, 119)
Tyrosine	Carboxylic acid (amino acid)	NMR[117, 120, 121]	EBC[117, 120], Urine[121]	Children[121], Adults[117, 120]	(117, 120, 121)
Taurine	Organic sulfonic acid (sulfur amino acid)	MS[115, 116]	Plasma[115], Serum[116]	Adults[115, 116]	(115, 116)
Butyrate	Fatty acyls (fatty acid)	NMR[120, 122]	EBC[120, 122]	Children[122], Adults[120]	(120, 122)
Acetate	Carboxylic acid	NMR[117, 118, 120, 122]	EBC[117, 120, 122], Serum[118]	Children[122], Adults[117, 118, 120]	(117, 118, 120, 122)
Formate	Carboxylic acid	NMR[117, 118, 120, 122]	EBC[117, 120, 122], Serum[118]	Children[122], Adults[117, 118, 120]	(117, 118, 120, 122)
Propionate	Carboxylic acid	NMR[117, 120, 122]	EBC[117, 120, 122]	Children[122], Adults[117, 120]	(117, 120, 122)
Glucose	Organooxygen compound (carbohydrate)	NMR[118, 120]	EBC[120], Serum[118]	Adults[118, 120]	(118, 120)
Ethanol	Organooxygen compound (alcohol)	NMR[117, 120]	EBC[117, 120]	Adults[117, 120]	(117, 120)
Methanol	Organooxygen compound (alcohol)	NMR[117, 118, 120, 122]	EBC[117, 120, 122], Serum[118]	Children[122], Adults[117, 118, 120]	(117, 118, 120, 122)
Urocanate	Azole (imidazole)	MS[123], NMR[117]	EBC[117], Urine[123]	Children[123], Adults[117]	(117, 123)

Legend: * - Classification according to "The human metabolome database" (http://www.hmda.ca, accessed in April 2018); EBC – exhaled breath condensate; MS – mass spectrometry; NMR – nuclear magnetic resonance.

Metabolites level changes depended on the analyzed matrix. For instance, low arginine plasmatic levels have been found in asthmatics[118], but this metabolite levels were higher in EBC of patients with asthma than healthy subjects[117]. More interestingly, these studies suggested that several metabolic pathways were altered in asthma. This preliminary data supported that further insights could contribute to increased knowledge in asthma. Despite very reductive, Figure 6 aims to represent the main metabolic pathways suggested to be significantly altered in asthma in at least two independent untargeted metabolomics studies in humans and also supported by targeted analyses. In particular, there was considerable consistency in identifying amino acids metabolism as significant. Amino acids can have antioxidant functions; in particular glycine, glutamine and glutamate may have potentially protective effects, whereas phenylalanine can have adverse effects[108]. Oxidative stress (resulting from an imbalance between oxidants and antioxidants in favor of oxidative states) has a significant role in asthma pathophysiology and lung damage[124]. Oxidized compounds that significantly distinguished asthmatics from healthy subjects have been identified in untargeted metabolomics studies[125]. Metabolic pathways associated with oxidative stress in asthma involved not only amino acids, including essential components in glutathione metabolism, but also lipids peroxidation[126]. The influence of the microbiome on asthma pathogenesis has recently gained much interest with novel evidence being added, namely regarding short chain fatty acids produced by intestinal microbiota and its role in oxidative stress and inflammation[127]. Changes in the energy metabolism with increased tricarboxylic acid-cycle metabolism have been associated with an enhanced requirement for energy in asthma exacerbations and uncontrolled asthma, with a more hypoxic, acidic and oxidizing environment. In this setting, purine metabolism has also been identified in untargeted metabolomics studies in humans. Although adenosine is well-known for its bronchoconstrictor and inflammatory effects, not only adenosine but also other related molecules have been detected as significantly altered in asthma, including deoxyadenosine, adenosine monophosphate and inosine[114, 115, 117]. Finally, metabolites related to the epigenetic pathways were also reported[117, 118]. In particular, epigenetic methylation has been suggested to skew immune responses towards a type 2 inflammation phenotype[128, 129].

There were also a number of non-replicated results. However, current evidence is insufficient to determine whether these represent spurious findings or reflect the substantial heterogeneity in the different studies. The metabolome can highly depend on the sample source, the analytic technique, its intrinsic coverage and data analysis, together with study design, besides the target population, particularly in complex heterogeneous diseases such as asthma. The primary aim of most metabolomics studies was to examine the differences between asthma cases and healthy controls, with a very few number of studies examining particular clinical asthma phenotypes. However, exploring the molecular profiling of clinically well-defined asthma phenotypes may reduce heterogeneity and potential bias.

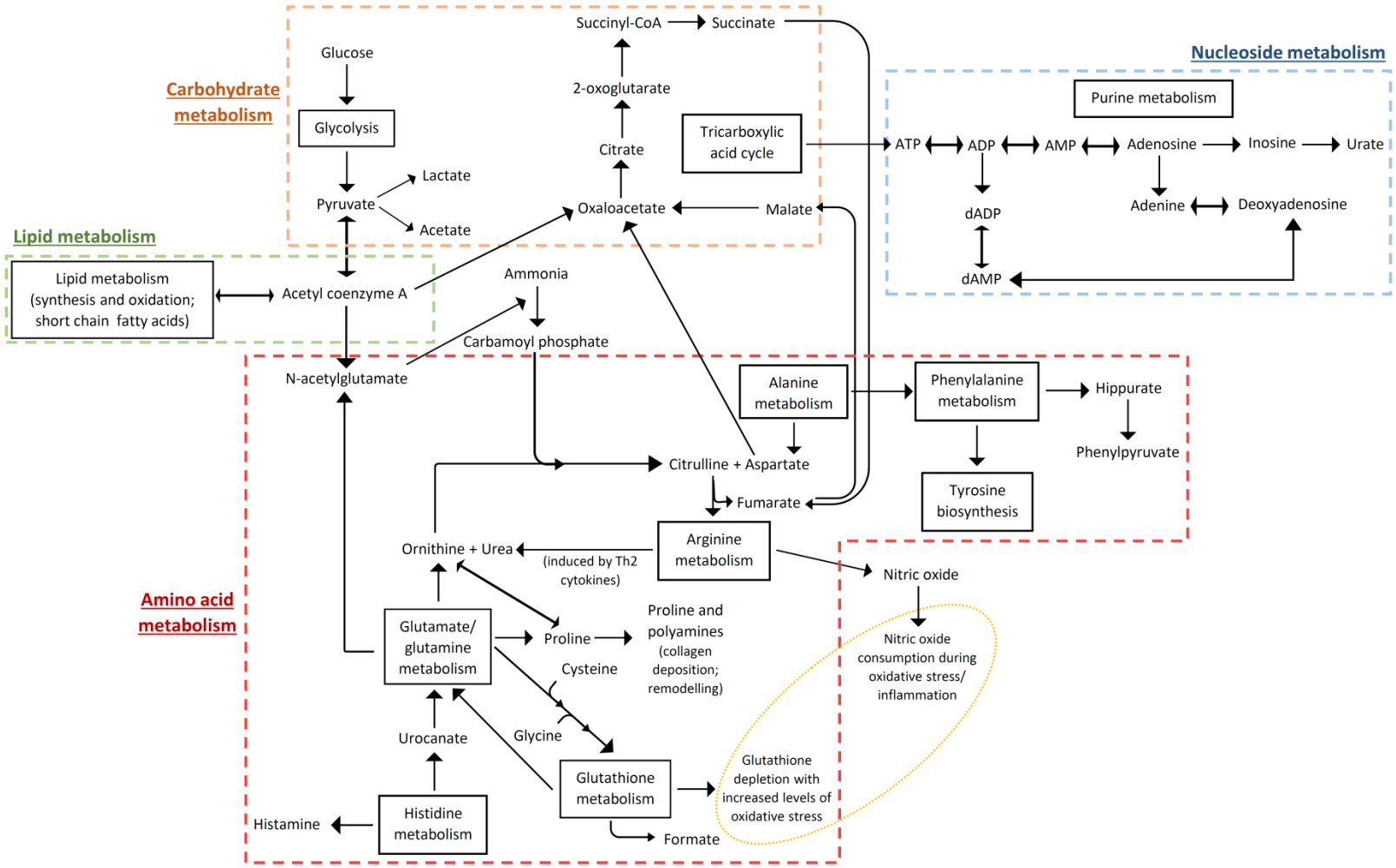


Figure 6 – Simplified example of metabolic pathways suggested to be altered in asthma in at least two independent untargeted metabolomics studies in humans (the net utilization or production of metabolites is dependent on cell type and location in the cell; arrows do not necessarily represent direct metabolism) Legend: ATP – adenosine triphosphate; ADP – adenosine diphosphate; AMP – adenosine monophosphate; dADP – deoxyadenosine diphosphate; dAMP – deoxyadenosine monophosphate; Th2 – T helper 2

Chapter 2: Aims

The main global objective of this dissertation was to contribute to unveil features for early diagnosis and recognition of asthma in the global airways disease context, advancing in the integrated control assessment and exploring innovative strategies for the molecular characterization of allergic rhinitis and asthma to step forward into endotypes, ultimately aiming to reduce the multimorbidity associated burden.

In particular, the original work aimed to address existing unmet needs in asthma and its link with rhinitis:

- nationwide, estimating asthma prevalence and analyzing its association with rhinitis in particularly vulnerable, internationally data-lacking populations – the children and the elderly *(Outputs 1 to 3)*;
- clinically, identifying multidimensional early childhood wheezing clinical phenotypes related to asthma persistence until adolescence *(Output 4)*;
- functionally, analyzing the association between nasal airflow and lower airway patency, together with the subjective evaluation of allergic rhinitis and asthma control in school-aged children *(Output 5)*.
- molecularly, exploring broad, data-driven differentiating metabolic features of childhood allergic rhinitis and asthma multimorbidity in non-invasively collected samples *(Outputs 6 and 7)*;

Specific primary aims:

Output 1: To estimate the prevalence of current asthma prevalence and asthma-like symptoms in the pediatric population (<18 years) living in Portugal.

Output 2: To estimate the prevalence of rhinitis in the population aged 65 years or above living in mainland Portugal and to classify rhinitis in this age group.

Output 3: To estimate the prevalence of physician-diagnosed asthma in the population aged 65 years or above living in mainland Portugal and to analyze its association with the presence and classification of rhinitis.

Output 4: To identify and characterize “hypothesis-free” multidimensional early childhood wheezing phenotypes related to asthma persistence in adolescence.

Output 5: To analyze the association between PNIF and (1) lower airway patency in children with allergic rhinitis and asthma and healthy children and (2) allergic rhinitis and asthma control subjective evaluation.

Output 6: To uncover “hypothesis-free” salivary and urinary differentiating metabolic features in children with allergic rhinitis and asthma multimorbidity compared to healthy children.

Output 7: To assess potential exogenous contaminants in EBC metabolic profiling.

Secondary aims can be found in each article.

Chapter 3: Methods

In order to address these aims, distinct methodologies were used:

- Cross-sectional, population-based, nationwide surveys of citizens living in Portugal, using standardized procedures applied by telephone interview (*Output 1*) and face-to-face interview (*Outputs 2 and 3*). The questionnaires included information on asthma and rhinitis allowing the analysis of the association between these two conditions.
  In *Output 1*, data from all individuals aged below 18 years who participated in the INAsma study (representative sample of all aged citizens in Portugal) were analyzed. The inclusion of 716 children allowed estimation of current asthma prevalence with an error of 2% (precision) and 95% confidence interval.
  In *Outputs 2 and 3*, the target population was the population aged 65 years or above living in mainland Portugal. The primary endpoint was rhinitis prevalence and a representative sample was analyzed, including a total of 3678 responders (*Output 2*). This sample allowed the estimation of asthma prevalence with an error <0.9% (precision) in 95% confidence interval (*Output 3*).
- Prospective cohort study of children aged below 7 years with recurrent wheezing (*Output 4*). The cohort included 308 children that were systematically evaluated at three, eight and 13 years of follow-up. Multivariable logistic regression models for persistent asthma in adolescence were developed based on questionnaires and skin prick tests data. Phenotypes were identified with k-means cluster analysis of variables selected with the logistic regression analysis and compared for predicting asthma prevalence, use of control treatments and lung function in childhood and adolescence.
- Cross-sectional, case-control study of 65 school-aged children with allergic rhinitis and asthma and 24 healthy children (matched for age and gender), with laboratorial assessments.
  a) Respiratory functional laboratory: Sequential assessments of PNIF before and after nasal decongestion and spirometry with bronchodilation test were performed. The CARATkids was used for the subjective control assessment in children with allergic rhinitis and asthma. Associations were investigated by multiple linear regression models (*Output 5*).
  b) Analytical laboratory: Untargeted metabolomics analysis of urine and saliva samples collected from each child was performed using NMR spectroscopy. Spectroscopic and clinical data were subjected to statistical analysis including multivariable and univariable approaches (*Output 6*).
     Additionally, EBC was collected from seven volunteers, together with room air samples collection. All samples were analyzed using NMR spectroscopy and the resulting spectra were compared (*Output 7*).

Details on each study methodology can be found in each article.

Chapter 4: Results / Publications

Part 1: Asthma prevalence and its association with rhinitis in the extremes of life

Output 1a:

Asthma-like symptoms, diagnostic tests, and asthma medication use in children and adolescents: a population-based nationwide survey

Publication type:	Original contribution
Authors:	Manuel Ferreira-Magalhães, Ana Sá-Sousa, Mário Morais-Almeida, Helena Pité, Luís Filipe Azevedo, Maria Inês Azevedo, António Bugalho-Almeida, João Almeida Fonseca
Journal:	Journal of Asthma
Publication year:	2016
Conception and design:	João Almeida Fonseca, Mário Morais-Almeida, António Bugalho-Almeida, Luís Filipe Azevedo
Provision of study materials and participants:	João Almeida Fonseca, Mário Morais-Almeida
Collection and assembly of data:	João Almeida Fonseca, Mário Morais-Almeida, Ana Sá-Sousa
Data statistical analysis:	Ana Sá-Sousa, Manuel Ferreira-Magalhães, Luís Filipe Azevedo, João Almeida Fonseca
Manuscript draft writing:	Manuel Ferreira-Magalhães
Data interpretation and manuscript review:	All authors
Final approval of manuscript:	All authors
Funding:	Sociedade Portuguesa de Alergologia e Imunologia Clínica; Sociedade Portuguesa de Pneumologia; Fundação Ciência e Tecnologia – Harvard Medical School Portugal (HMSPIDSIM/SIM/0018/2009)
Ethical Committee approval:	Ethics Committee of Centro Hospitalar de São João, Porto, Portugal.

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Asthma-like symptoms, diagnostic tests, and asthma medication use in children and adolescents: a population-based nationwide survey

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Abstract

Objective: This study aimed to estimate the prevalence of asthma-like symptoms, current asthma (CA), asthma diagnostic tests, and inhaled medication use in a nationwide pediatric population (<18 years). *Methods*: Pediatric-specific data from a cross-sectional, population-based telephone survey (INAsma study) in Portugal were analyzed. CA was defined as lifetime asthma and (1) wheezing, (2) waking with breathlessness, or (3) asthma attack in the previous 12 months, and/or (4) taking asthma medication at the time of the interview. *Results*: In total, 716 children were included. The prevalence of asthma-like symptoms was 39.4% [95% confidence interval (95% CI): 35.7–43.3]. The most common symptoms were waking with cough (30.9%) and wheezing (19.1%). The prevalence of CA was 8.4% (95% CI: 6.6–10.7). Among children with CA, 79.9% and 52.9% reported prior allergy testing and pulmonary function testing (PFT), respectively. Inhaled medication use in the previous 12 months was reported by 67.6% (reliever inhalers, 40.1%; controller inhalers, 41.5%). Those who only used inhaled reliever medications experienced more asthma attacks [odds ratio (OR): 2.69]. Significantly fewer children with CA living in rural areas than those living in urban areas had undergone PFT or used inhaled medication (OR: 0.06 for PFT, 0.20 for medication]. *Conclusions*: The prevalence of CA in the Portuguese pediatric population was 8.4%. Only half of children with CA had ever undergone PFT; more than half did not use controller inhalers, and those who only used reliever inhalers reported more asthma attacks. These findings suggest that asthma management has been substandard, mainly in rural areas.

Keywords

Cough, epidemiology, pediatrics, prevalence, respiratory function tests, treatment, wheezing

History

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Introduction

Asthma is one of the most common chronic diseases in children [1]. According to the Global Initiative for Asthma, asthma is a heterogeneous disease characterized by variable symptoms [2]. Composite measures to define asthma are increasingly necessary to obtain more accurate epidemiological prevalence estimates [3], and population-based studies are important for the assessment of these estimates [4].

The results of the 20-year International Study of Asthma and Allergies in Childhood (ISAAC) have changed our knowledge about the worldwide prevalence of asthma and related symptoms [5]. The ISAAC included some Portuguese cities [6], but neither the ISAAC nor other studies estimated the prevalence of asthma across all pediatric ages (<18 years) or among a representative nationwide sample in Portugal [7–10]. In fact, to the best of our knowledge, very few studies have estimated the prevalence of asthma in this age range using nationwide samples [11–13]. Moreover, different asthma definitions are presented in the literature, making it difficult to accurately compare estimates between studies [3,14]. The patterns of asthma-like symptoms may also differ among various pediatric ages. However, no data have been published on the prevalence of specific asthma symptoms, other than wheezing, in all pediatric ages.

A diagnosis of asthma is based on symptoms and confirmation of variable expiratory flow limitation, making pulmonary function testing (PFT) an important tool. A

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stepwise approach for asthma management takes the previous two features and medication, primarily inhaler use, into account [2]. Epidemiological assessment of asthma should also focus on data from the above-mentioned asthma features closely linked to diagnosis and management.

In 2010, we conducted the First Portuguese National Asthma Survey (INAsma) [15]. In the present paper, we focus on the results obtained from the INAsma pediatric population. Specifically, our primary aim was to estimate the prevalence of asthma-like symptoms and current asthma (CA) in the Portuguese pediatric population (<18 years). Our secondary aims were to describe the differences in CA prevalence estimates according to different definitions to allow for comparisons with other studies and to assess the proportion of the use of diagnostic tests and inhaled medication among children with CA.

Materials and methods

Study design and participants

The INAsma was a population-based, nationwide, cross-sectional survey applied by telephone interview in Portugal in 2010. The study methodology has been previously described [15,16]. The INAsma target population was the Portuguese general population, and the available population included citizens of all ages living in households with a landline telephone. A cluster sampling design was used to obtain a representative sample of the population (detailed in the Supplementary file). Briefly, all municipalities were used as natural strata, and a sample of households was then selected with a probability proportional to the municipality population as estimated in the 2001 National Census. Households were randomly selected from the 2010 residential White Pages telephone directory. Four different lists of telephone numbers were randomly selected for each municipality to allow for substitution of nonresidential telephone numbers. In total, 24412 telephone numbers were retrieved. One participant (the last person to have had a birthday) was randomly selected from each household. In this study, we considered all included individuals aged <18 years (henceforth referred to as ''children'' for simplification).

This study was reviewed and approved by the Ethics Committee of Centro Hospitalar de São João, Porto, Portugal. Informed consent was obtained from the parent(s) or legal guardian(s) of the children included in this study. We followed the STROBE statement for reporting observational studies [17].

Instruments and data collection

The instrument for data collection was the Portuguese version of the questionnaire used in the GA^{2}LEN survey, which includes the European Community Respiratory Health Survey questions on asthma symptoms [18]. Several questions were added regarding socio-educational variables, medications, and diagnostic test use for asthma. Data were collected by computer-assisted telephone interviews from March to May 2010. Selected participants directly answered the questionnaire. However, in accordance with Portuguese legislation, when the selected individual was younger than 15 years old, the respondent was the usual and most knowledgeable caregiver. To minimize potential bias in data collection, several quality-assurance measures were followed (Supplementary file) [15].

Variables

Asthma definition variables were defined based on previous studies [3,5]. The outcomes were defined as follows: *Lifetime asthma (LA)*: positive answer to ''Have you ever had asthma?''; *CA*: LA plus at least one of the following four features: (1) wheezing, (2) waking with breathlessness, (3) having an asthma attack in the last 12 months, or (4) taking asthma medication at the time of the interview; *Nocturnal symptoms*: at least one of the three symptoms in the last 12 months: waking with breathlessness, waking with chest tightness, or waking with coughing; *Asthma-like symptoms*: at least one of the three symptoms in the last 12 months: wheezing, nocturnal symptoms, or asthma attacks (wheezing was further described as wheezing with breathlessness or wheezing without a cold); *PFT*: positive answer to ''Have you ever undergone any test to assess your lung function?''; *Skin prick tests*: positive answer to ''Have you ever undergone any skin test to assess your allergies?''; *Blood allergy tests*: positive answer to ''Have you ever undergone any blood test to assess your allergies?''; *Any asthma medication at present*: positive answer to ''Are you currently taking medication (inhaled, nebulized, or oral) for asthma/breathlessness?''; *Inhaled therapy in the last 12 months*: positive answer to ''In the last 12 months, did you take any inhaled medication for asthma/breathlessness?'' Further characterization of inhaled treatment was based on auto-reported medication: *Allergic rhinitis*: Positive answer to ''Do you have any nasal allergies, including hay fever?''; *Atopic dermatitis/eczema*: Positive answer to ''Have you ever had eczema or skin allergy?''; *Food allergy*: Positive answer to ''Have you been diagnosed with food allergy by a doctor?''; and *Environmental tobacco smoke*: Positive answer to ''Does anyone smoke inside your home?''. *Municipality* was classified as urban if the residence was located in a municipality with at least one city (cities must have at least 8000 citizens), according to Portuguese legislation.

Sample size and statistical analysis

The study sample size was calculated for the total Portuguese population and took into account the two phases of the INAsma project [15]. Thus, for the general all-age population, we estimated an *a priori* asthma prevalence of 6% and a proportion of controlled asthma of 20%. Assuming a 20% loss to follow-up, at least 665 patients with asthma were required. Within these premises, and assuming a margin of error of 0.65%, our study required a sample of at least 6000 persons from the general Portuguese population willing to participate in the first phase of the INAsma. The inclusion of 716 children allowed us to estimate CA prevalence with an error margin of 0.02 and a 95% confidence level.

The sample estimates were weighted according to the 2001 National Census aiming for generalization to the target population [19]. A sequential simple random sampling method without replacement was used to select a random

sample of households with a landline telephone within each stratum (municipality) and then one random eligible household resident. Two types of weights were used: (1) prestratification weights to adjust for the sampling design and (2) poststratification weights to adjust for the true sex and age distribution of the target population, thus partially correcting for nonresponse and noncoverage bias.

Statistical analyses were weighted for the Portuguese pediatric population and performed using the complex samples tool of SPSS version 21 (SPSS IBM, New York, NY, USA), with the exception of the participants' characteristics description, which was performed without weighting procedures. Categorical variables were described using absolute and relative frequencies with 95% confidence intervals (95% CIs); comparisons were performed with Pearson's chi-squared test. Univariate logistic regression models for outcomes were developed using independent variables as risk/predictive factors. Multivariate logistic regression models were developed to adjust for possible confounders. Results of multivariate logistic regression models were presented as odds ratios (ORs) with 95% CIs. A p value of <0.05 was considered statistically significant.

Results

Participants

The corrected response rate of the INAsma was 50%. Of the 6003 participants in the INAsma survey, 716 (11.9%) were children. A flowchart of all participants is shown in Figure S1. One-fifth (21.4%) of the children were younger than 6 years of age, and 39% were 13–17 years old; most (80.6%) were from urban areas. The children's characteristics are summarized in Table 1.

Prevalence of asthma-like symptoms

The prevalence of asthma-like symptoms among Portuguese children was 39.4% (95% CI, 35.7–43.3) (Table 2). Asthma-like symptoms were highest in children aged <6 years (47.9%; 95% CI, 40.0–56.0) and lowest in those aged 13–17 years (31.8%; 95% CI, 26.3–37.8; $p=0.005$) (Table S1). Waking during the night with cough was the most prevalent symptom (30.9%; 95% CI, 27.5–34.5), followed by wheezing (19.1%; 95% CI, 16.4–22.1).

The prevalence of nocturnal symptoms was higher in children aged <6 years (39.8%; 95% CI, 32.1–48.1) than in those aged 13–17 years (OR: 0.61; 95% CI, 0.40–0.94), although the prevalence was not significantly different from that of children aged 6–12 years (OR: 0.84; 95% CI: 0.55–1.28). The prevalence of wheezing was highest in children aged <6 years (31.0%; 95% CI: 24.4–38.5) and significantly decreased with age (OR: 0.46; 95% CI: 0.30–0.70 in the 6- to 12-year-old age group; and OR: 0.24; 95% CI: 0.15–0.40 in the 13- to 17-year-old age group) (Figure 1). Among children who reported wheezing in the last 12 months, the symptoms of wheezing without a cold and wheezing with breathlessness were higher in children aged 6–12 years than in those aged <6 years ($p<0.001$) (Figure 2).

The presence of rhinitis and eczema was associated with a higher prevalence of having any asthma-like symptom (OR: 3.67; 95% CI: 2.52–5.39 for rhinitis; and OR: 2.11; 95% CI: 1.51–2.94 for eczema). Supplementary information on asthma-like symptoms is presented in Tables S1 and S2.

Asthma prevalence

The prevalence of LA was 11.2% (95% CI: 9.1–13.7), and that of CA was 8.4% (95% CI: 6.6–10.7). No statistically significant differences were found in the prevalence of CA according to region or other sociodemographic variables (Table 1). A higher risk of CA was found in children with rhinitis and eczema (OR: 5.20; 95% CI: 3.05–8.85 for rhinitis; and OR: 4.17; 95% CI: 2.41–7.19 for eczema). Table 3 shows an overview of different asthma definitions according to the inclusion of different proxies of asthma prevalence.

Diagnostic tests and medication for asthma

PFT and allergy tests (skin prick tests and/or blood allergy tests) were performed in 12.6% (95% CI: 10.3–15.2) and 28.3% (95% CI: 24.9–31.9) of children, respectively. About half of children with CA aged ≥6 years had undergone PFT (52.9%; 95% CI: 46.0–59.8), and this prevalence did not change if they had had an asthma attack in the previous 12 months. Children with CA who lived in rural areas were less likely to report diagnostic testing than those from urban areas (OR: 0.06; 95% CI: 0.04–0.09 for PFT; and OR: 0.39; 95% CI: 0.22–0.69 for allergy tests) (Table 4).

Among children with CA, the frequency of any asthma medication at the time of the interview was 76.4% (95% CI: 64.4–85.3). Inhaled reliever and controller therapy were used in the previous 12 months by 40.1% (95% CI: 28.5–53.0) and 41.5% (95% CI: 29.6–54.5) of children with CA, respectively (Table S3). Children who reported using only inhaled reliever medication in the previous year, without any inhaled controllers, were more likely to experience an asthma attack in the same time period (OR: 2.69; 95% CI: 1.24–5.84). Children with CA from rural areas were less likely to report using any inhaled therapy in the previous 12 months (OR: 0.20; 95% CI: 0.09–0.43).

Discussion

A high prevalence of asthma-like symptoms was found in the Portuguese pediatric population (39.4%; 95% CI: 35.7–43.3), and the prevalence of CA was 8.4% (95% CI: 6.6–10.7). This study showed that less than half of children with CA had been on inhaled controller medication in the previous 12 months and that only 52.9% of those aged ≥6 years had undergone PFT.

This study was the first population-based nationwide epidemiological study aiming to estimate the asthma prevalence in Portugal among all municipalities and pediatric ages. It included a large representative sample of the Portuguese population, allowing us to estimate the prevalence of asthma-like symptoms, CA, diagnostic tests, and medication use in asthmatic children. Nationwide studies on the prevalence of asthma across the entire pediatric population have only been previously performed in Germany and the United States [11–13]; no previous study has reported the prevalence of asthma-like symptoms across all pediatric ages. A multinational study of children aged 1–5 years showed that

Table 1. Participants' characteristics in a pediatric sample.

	Total participants, *n* (%)	Current asthma % (95% CI)	Odds ratio[a] (95% CI)
All pediatric Population	716 (100.0)	8.4 (6.6–10.7)	
Gender			
Female	270 (37.7)	6.7 (4.3–10.1)	1.00 (Ref)
Male	446 (62.3)	10.1 (7.5–13.4)	1.59 (0.92–2.75)
Age groups			
0–5 years	153 (21.4)	6.5 (3.5–11.6)	1.00 (Ref)
6–12 years	284 (39.7)	9.7 (6.8–13.8)	1.53 (0.73–3.23)
13–17 years	279 (39.0)	8.7 (5.9–12.5)	1.36 (0.64–2.90)
National region			
North	284 (39.7)	7.2 (4.6–11.1)	1.00 (Ref)
Centre and Islands	179 (25.0)	9.7 (6.3–14.7)	1.35 (0.67–2.74)
South	253 (35.3)	8.7 (5.7–12.9)	1.21 (0.61–2.38)
Municipality			
Urban	577 (80.6)	8.0 (5.9–10.8)	1.00 (Ref)
Rural	139 (19.4)	9.8 (6.6–14.3)	1.12 (0.59–2.11)
ETS[b]			
No	454 (63.4)	8.2 (5.9–11.3)	1.00 (Ref)
Yes	262 (36.6)	8.8 (6.1–12.6)	1.06 (0.61–1.84)
Allergic rhinitis			
No	558 (77.9)	5.0 (3.4–7.4)	1.00 (Ref)
Yes	158 (22.1)	22.3 (16.6–29.2)	5.15 (3.02–8.78)*
Eczema			
No	517 (72.2)	5.0 (3.4–7.4)	1.00 (Ref)
Yes	199 (27.8)	18.3 (13.6–24.2)	4.08 (2.35–7.08)*
Food allergy			
No	676 (94.4)	8.3 (6.4–10.8)	1.00 (Ref)
Yes	40 (5.6)	9.6 (5.4–16.4)	0.94 (0.45–1.95)

Weighted proportion and odds ratio (95% confidence intervals) of current asthma according to sociodemographic and clinical variables.
[a]Adjusted odds ratio for gender, age, region, municipality, and ETS.
[b]Environmental tobacco smoke.
*Statistically significant.

Table 2. Weighted prevalence of asthma-like symptoms in the previous 12 months in the Portuguese pediatric population.

		Current asthma	
	Total population % (95% CI)	Yes % ($n=66$)	No % ($n=750$)
Any symptom[a]	39.4 (35.7–43.3)	91.4 (82.4–96.1)	34.7 (30.9–38.6)
Wheezing	19.1 (16.4–22.1)	76.5 (65.4–84.9)	13.8 (11.4–16.6)
Wheeze w/o cold	8.2 (6.5–10.4)	49.2 (37.2–61.3)	4.5 (3.1–6.3)
Nocturnal symptoms	34.7 (31.1–38.5)	79.2 (68.0–87.3)	30.7 (27.0–34.6)
Waking with breathlessness	5.0 (3.6–7.1)	24.7 (16.1–35.9)	3.2 (2.0–5.3)
Waking with chest tightness	6.7 (5.1–8.8)	27.4 (17.8–39.6)	4.8 (3.4–6.8)
Waking with cough	30.9 (27.5–34.5)	68.7 (56.6–78.7)	27.4 (24.0–31.2)
Asthma attack	3.7 (2.6–5.4)	44.4 (32.6–56.9)	0.0 (0.0–0.0)

[a]Wheezing *or* waking with breathlessness *or* waking with chest tightness *or* waking with cough *or* asthma attacks.

approximately one-third had wheezing, coughing, or breathlessness [20], which is lower than our observation in children aged <6 years (47.9%). However, the time period considered in that study was only 6 months, while the time period in our study was 12 months. Two recent Portuguese studies reported estimates of wheezing in preschoolers (24.5% and 27.5%) [21,22], similar to our results. Mvula et al. [23] found that 27.3% of children aged 5–18 years had cough at night, apart from cold or chest infection. Our question did not distinguish different causes of cough and association to colds; however, our prevalence among children aged 0–17 years (30.9%) is similar to that reported by Mvula et al. [23].

The United States National Center for Health Statistics reported a 5.4% prevalence of asthma attacks among children aged 0–17 years [24]. The slightly lower prevalence among Portuguese children (3.7%) may be related to differences in the definition of asthma attacks in the two populations.

Differences in asthma-like symptom patterns in various age subgroups

The prevalence of wheezing and nocturnal symptoms decreased with age (Figure 1). However, after the age of 5 years, most children with wheezing exhibited wheezing without a cold and wheezing with breathlessness (Figure 2). These results suggest that age influences asthma-like symptom patterns. These findings are in agreement with previous studies suggesting that wheezing

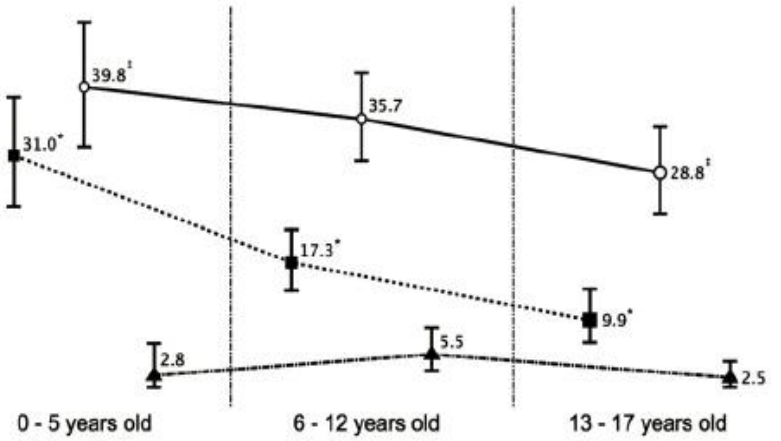

Figure 1. Weighted prevalence of asthma-like symptoms within the previous 12 months, according to age group (%, 95% CI). Symbols represent prevalence of: ◯ nocturnal symptoms (waking with breathlessness *or* waking with chest tightness *or* waking with cough); ■ wheezing; ▲ asthma attack. *Difference between age groups with $p<0.001$; ‡Difference between age groups with $p<0.001$.

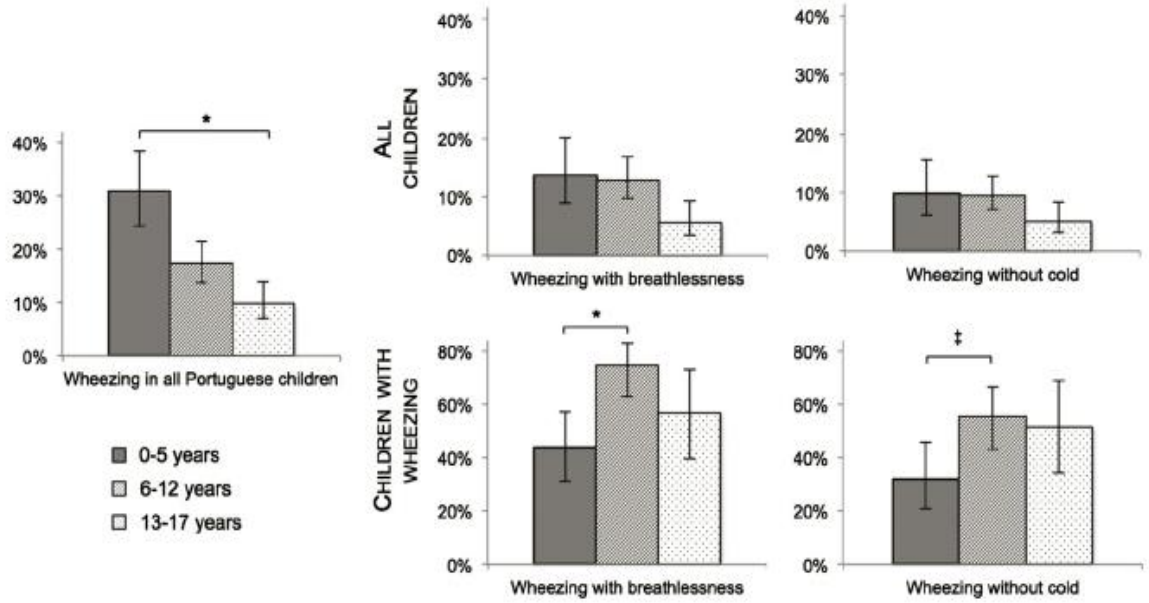

Figure 2. Weighted prevalence of wheezing with breathlessness and wheezing without a cold in the Portuguese pediatric population. *$p<0.001$, ‡$p=0.03$. Prevalence of wheezing in all Portuguese children: 31.0% in 0- to 5-year age group (OR: 1.00); 17.3% in 6- to 12-year age group (OR: 0.46; 95% CI: 0.30–0.72); 9.9% in 13- to 17-year age group (OR: 0.25; 95% CI: 0.15–0.41). Prevalence of wheezing with breathlessness in all Portuguese children: 13.6% in 0- to 5-year age group; 12.8% in 6- to 12-year age group; 5.7% in 13- to 17-year age group. Prevalence of wheezing without a cold in all Portuguese children: 9.9% in 0- to 5-year age group; 9.5% in 6- to 12-year age group; 5.1% in 13- to 17-year age group. Prevalence of wheezing with breathlessness among all children who wheezed: 43.7% in 0- to 5-year age group (OR: 1.00); 74.4% in 6- to 12-year age group (OR: 3.73; 95% CI: 1.79–7.78); 57.0% in 13- to 17-year age group (OR: 1.71; 95% CI: 0.83–3.50). Prevalence of wheezing without a cold among all children who wheezed: 31.9% in 0- to 5-year age group (OR: 1.00); 55.2% in 6- to 12-year age group (OR: 2.64; 95% CI: 1.27–5.48); 51.7% in 13- to 17-year age group (OR: 2.28; 95% CI: 0.96–5.43).

in younger children is more closely related to immature airways and viral respiratory illnesses [25], whereas older children tend to exhibit wheezing even without respiratory infections.

Asthma prevalence

There is no consensus on the epidemiologic definition of CA, which leads to hardly comparable differences in reported prevalence estimates, depending on the definition used [3,14]. Therefore, we presented estimates using different combinations of asthma proxies (Table 3). The definition we chose includes the less frequent nocturnal symptom, waking with breathlessness; the term asthma attack to improve specificity; and asthma medication, because children with asthma may not experience symptoms when controlled with medication, and medication is a known valid proxy for asthma prevalence [26,27]. By using definitions of prevalence used in different studies, our prevalence estimates varied from 6.3% (LA *plus* wheezing in the last year) and 9.1% (LA *plus* any asthma-like symptom in the last year *or* CA medication). These differences highlight the importance of setting standardized definitions to allow for comparisons between studies.

Table 3. Weighted Asthma prevalence in the Portuguese pediatric population, using different asthma proxies.

	Population % (95% CI)	Total weighted population (2,066,953)
Lifetime asthma	11.2 (9.1–13.7)	230,886
Current asthma (with symptoms in the previous 12 months)		
Wheezing w/o cold	8.2 (6.5–10.4)	169,991
Lifetime asthma + wheezing	6.4 (4.8–8.6)	132,848
Lifetime asthma + wheezing *OR* waking with breathlessness	7.0 (5.3–9.1)	143,958
Lifetime asthma + wheezing *OR* asthma attack *OR* waking with breathlessness	7.2 (5.5–9.4)	149,199
Lifetime asthma + wheezing *OR* waking with breathlessness *OR* waking chest tightness *OR* waking cough	8.3 (6.5–10.6)	171,829
Lifetime asthma + wheezing *OR* asthma attack *OR* waking with breathlessness *OR* current asthma medication[a]	8.4 (6.6–10.7)	173,681
Lifetime asthma + wheezing *OR* asthma attack *OR* waking with breathlessness/chest tightness/cough	8.4 (6.6–10.7)	173,963
Lifetime asthma + wheezing *OR* asthma attack *OR* current asthma medication *OR* waking with breathlessness/chest tightness/cough	9.1 (7.2–11.5)	188,819

[a]Definition used as *current asthma*.

Table 4. Weighted proportions (95% confidence interval) of pulmonary function tests, allergy tests (skin or blood), and inhaled medication use in Portuguese children with current asthma.

	PFT[a] ever % (95% CI)	Allergy tests[b] ever % (95% CI)	Inhaled meds[c] in the last 12 months % (95% CI)
All	45.7 (33.8–58.1)	79.9 (67.9–88.2)	67.6 (58.4–75.7)
Gender			
Female	36.9 (26.0–49.3)	87.0 (67.5–95.5)	74.9 (70.7–79.3)
Male	51.4 (41.3–61.4)	75.3 (70.2–79.8)	62.9 (49.1–74.9)
Age groups			
0–5 years old	**21.9 (13.5–33.6)**	**48.4 (27.1–70.3)**	72.1 (38.7–91.4)
6–12 years old	**46.4 (40.2–52.7)**	**97.1 (96.8–97.5)**	72.5 (64.8–79.1)
13–17 years old	**61.5 (47.4–73.9)**	**79.4 (69.0–87.0)**	58.1 (47.4–68.1)
National region			
North	44.0 (29.6–59.4)	**93.3 (82.4–97.7)**	57.9 (36.5–76.8)
Centre and Islands	38.6 (29.5–48.5)	**75.6 (75.2–75.9)**	69.8 (69.3–70.2)
South	53.9 (41.0–66.2)	**71.3 (56.2–82.7)**	74.7 (61.2–84.7)
Municipality			
Urban	**57.6 (46.9–67.7)**	**83.9 (74.8–90.1)**	**76.4 (63.4–85.9)**
Rural	**7.3 (7.2–7.3)**	**67.0 (66.6–67.5)**	**39.2 (29.5–49.9)**
Allergic rhinitis			
No	43.8 (33.8–54.4)	**71.4 (59.5–81.0)**	67.6 (51.8–80.1)
Yes	47.4 (38.9–56.1)	**87.7 (84.0–90.7)**	67.7 (59.5–74.9)
Food allergy			
No	46.2 (38.4–54.2)	**78.6 (72.0–84.0)**	67.6 (57.8–76.1)
Yes	37.6 (37.6–37.6)	**100.0 (n/a)**	67.8 (67.8–67.8)

[a]PFT, pulmonary function tests. [b]Allergy tests: blood and/or skin tests. [c]Inhaled medications: inhaled medication use. n/a: not applicable.
Bold values are statistically significant differences between row subgroups.

Our estimate of LA is based on the same question of ISAAC. The estimate in the 6- to 12-year-old age group (12.3%) was higher than that in the 6- to 7-year-old age group in the Portuguese ISAAC survey (9.8%) [6], while our estimate for the 13- to 17-year-old age group (13.9%) was similar to that in the 13- to 14-year-old age group in the ISAAC survey (13.8%). In preschoolers, two recent Portuguese studies had also reported estimates of physician asthma diagnosis similar to ours: 4.6% in both studies [21,22].

Schmitz et al. [11] reported a 4.7% prevalence of a doctor diagnose LA in the Germany population of 0- to 17-year-old using data from the 2003–2006 German Health Interview and Examination Survey for Children and Adolescents (KiGGS). This prevalence was much lower than ours and is in agreement with the estimated prevalence of LA among German children reported in the ISAAC, which was also much lower than that in Portugal (in Germany, 4.3% in 6- to 7-year-old children and 7.5% in 13- to 14-year-old children) [5]. Data from the 2010 United States National Health Interview Survey for children aged <18 years reported an estimated doctor diagnosed LA prevalence of 13.6% and CA prevalence of 9.5% [12,13], which are similar to our estimations.

Asthma symptoms other than wheezing

In children, epidemiological asthma definitions and clinical asthma phenotypes are primarily based upon wheezing [28]. However, Skytt et al. [29], based on data from the Copenhagen Prospective Study on Asthma in Childhood,

addressed the lack of validity of wheezing for asthma diagnosis in children aged 0–3 years. They argued for a global description of asthma symptoms to reduce the risk of misclassification of asthma in children. Wheezing-based asthma definitions are also limited by differences in the concept and perception of wheezing. Fernandes et al. [30] reported a lack of perception of the term ''wheezing'' among Portuguese parents and health professionals. To account for this problem, we used several synonyms of wheezing in Portuguese in wheezing-related questions. Additionally, the presence of wheezing was not mandatory in our definition of CA.

We suggest that questionnaires directed at children and adolescents should include different measures to comprehensively assess asthma characteristics (previous diagnosis, several symptoms, and medication), thus aiming to obtain precise estimates of asthma prevalence, especially in younger children. In any case, standardization of epidemiologic asthma definitions and their clinical validation are needed [3].

Diagnostic tests and medication for asthma

In spite of the recommendations to perform spirometry as part of asthma management [2], only about half of the Portuguese children with CA aged ≥6 years had undergone PFT. Similarly, fewer than half of Portuguese children with CA had used inhaled controller medications within the previous 12 months, even considering a CA definition based on several symptoms occurring in the last 12 months, like the one used in our study. Moreover, those who only used reliever inhaled medication in the previous year, without any controller inhalers, were twice as likely to have experienced an asthma attack in the same time period.

No differences in the prevalence of asthma or asthma-like symptoms were found between urban and rural municipalities; however, children living in rural areas were much less likely to have undergone PFT or allergy tests or used inhaled medication. In agreement with our data, a Greek study with school-aged children also showed that the prevalence of asthma diagnosis was the same between rural and urban areas despite findings on the narrowing of small airways in urban environments [31]. Disparities in health care for rural-dwelling patients with asthma have been reported, indicating that those with asthma who live in rural areas receive substandard care for asthma; however, there are limited direct data on these findings [32]. Taken together, our results and those of previous studies on diagnostic tests and medication use may be related to differences in health-care access and may suggest substandard asthma care in Portuguese children, especially in less urbanized areas.

Limitations

This study has several limitations. The survey was designed for the overall Portuguese population; however, the questions included in the questionnaire and the definitions used are similar to others used in international and Portuguese pediatric studies [33–35]. The use of the 2001 National Census for sampling could not account for the reduction in the number of births that occurred in the last decade; however, it was the most recently available data at the time of data collection. Also, the study's pediatric sample follows the characteristics of the Portuguese pediatric population characteristics [19]. The use of landline telephones excludes households without residential telephones; nevertheless, it allowed us to guarantee a stratified cluster sample by region. Answers were given by the main caregiver for participants <15 years old and by the adolescent when the participant was ≥15 years old. This may limit comparisons between these age groups; however, changes of parent-reported to auto-reported outcomes are recommended between school age and adolescence [23], and other studies on asthma prevalence in pediatric ages had similar methodology [11]. Finally, we could not obtain a clinical confirmation of the diagnosis; therefore, participants' misclassification can be expected, as in any similar survey.

Conclusion

Asthma-like symptoms were present in more than one-third of Portuguese children, and the prevalence of CA was 8.4%. Age influenced asthma-like symptoms patterns, and asthma prevalence varied according to different definitions. Therefore, using composite measures and reporting different operational estimates is advisable. Fewer than half of the children with CA had used inhaled controller medications in the previous year, and those who used inhaled relievers alone had experienced twice more asthma attacks. PFT were underused among asthmatic children. Also, the findings on low inhaled controller medication and PFT use were more prevalent in children living in rural areas.

Declaration of interest

The authors report no conflicts of interest. None of the authors received any honorarium, grant, or other form of payment to produce the manuscript. The authors alone are responsible for the content and writing of the paper.

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SUPPLEMENTARY FILE

Asthma-like symptoms, diagnostic tests and asthma medication use in children and adolescents: a population-based nationwide survey

INAsma STUDY DETAILS

Sample size

The sample size calculation was based on confidence intervals of proportions. Thus, for a prevalence of 6% and narrow error margin of 0.015, a sample of 1067 individuals was required. However, the sample size of this study was calculated considering the two phases of the research project. In the first phase, we aimed to estimate the current asthma prevalence in the Portuguese population. In the second phase, we aimed to estimate the proportion of patients with controlled asthma symptoms. Thus, in the second phase of the project, to estimate the proportion of patients with controlled asthma with a 3% margin of error and 95% confidence level, and assuming a proportion of 20% of patients with uncontrolled asthma in the population, we required a sample of at least 554 patients with asthma identified in the first phase of the project. Assuming a 20% loss to follow-up, we required at least 665 patients with asthma during the first phase. Within these premises, and assuming a prevalence of 6% in the Portuguese population and a margin of error of 0.65%, we required a sample of at least 6000 persons from the general Portuguese population willing to participate in the first phase of the project.

Participants

The target population was the Portuguese general population. The available population included all individuals living in Portugal in households with a landline telephone (sampling frame). To obtain a representative sample of the population, a stratified cluster sampling design was used.

First, all municipalities were used as natural strata; in each municipality, a sample of households with a landline telephone number was selected with a probability proportional to the municipality population as estimated in the 2001 National Census. The target number of households was set at 6103. The sample of households was derived from the directory listed in the residential White Pages from 2010. A list of all telephone numbers in a specific municipality was compiled to draw a sample of telephone numbers within that municipality. A sample of household telephone numbers was randomly selected from the whole list in each municipality. Because part of the selected telephone numbers were from companies or were not allocated, four lists were randomly selected for each municipality to allow for substitution of non-residential telephone numbers. In total, 24 412 telephone numbers were retrieved.

After the selection and identification of a residential number, one participant was randomly selected from each household. After identification of all residents in the household, the selected participant in each household was the last person to have had a birthday. When the selected individual was younger than 15 years, the respondent was the usual caregiver. Individuals were excluded if they did not understand spoken Portuguese or had cognitive or physical conditions that could hamper the interview. In the final 20% of the sample, an oversampling strategy of male adults and younger adults subjects was used to correct the common overrepresentation of female and older participants observed in the interim analysis.

Data collection

The main instrument used for data collection was the Portuguese version of the 21-item questionnaire used in the GA2LEN survey. This questionnaire includes the ECHRS questions on asthma symptoms. A few additional questions were added, mostly regarding socioeducational variables and the use of healthcare resources.

An experienced private company administered the questionnaire through computer-assisted telephone interviews performed by trained and experienced interviewers. Interviews were conducted from March to May of 2010, mostly from 17:00 to 22:00 h on weekdays and 11:00 to 22:00 h on weekends and holidays. No telephone number was abandoned until a minimum of 10 attempts had been made on different occasions. The interviews had a mean duration of 15 min.

To minimize other potential biases in data collection, several quality assurance measures were followed: interviewers were selected based on their previous experience with health-related data collection; each question was discussed in training sessions held between researchers and all interviewers; a research assistant was present during the setup, training, and daily work of the interviewers, motivating and checking compliance with standardized operational procedures; data validity was periodically verified soon after being collected, and custom statistic algorithms were used to detect extreme, illogical, and missing values; and the clarity of the questionnaire and its telephonic administration were assessed in a pilot study involving 25 individuals before starting the data collection.

Variables

Variables were defined as follows.

Nocturnal symptoms - at least one of 3 symptoms in the last 12 months: waking with breathlessness, waking with chest tightness or waking with coughing.

Asthma-like symptoms - at least one of 3 symptoms in the last 12 months: wheezing, nocturnal symptoms, or asthma attacks.

Lifetime asthma: Positive answer to "Have you ever had asthma?".

Current asthma: Lifetime asthma with at least one of 4: wheezing, waking with breathlessness or having an asthma attack in the last 12 months, or taking asthma medication at the time of the interview.

Pulmonary function tests - positive answer to "Have you ever done any test to assess your lung function?" (the interviewer further explained that "it refers to an exam that assesses the breathing capacity, i.e., spirometry, or blowing harder into a machine, or breathing inside a glass box, other than blowing quickly to a plastic tube or peak flow meter").

Skin prick tests - positive answer to "Have you ever done any skin test to assess your allergies?".

Blood allergy tests - positive answer to "Have you ever done any blood test to assess your allergies?".

Any asthma medication at the time of interview - positive answer to "Are you currently taking medication (inhaled, nebulized or oral) for asthma/breathlessness?".

Inhaled therapy in the last 12 months - positive answer to "In the last 12 months, did you take any inhaled and/or nebulized medication for asthma/breathlessness?". Further characterization of inhaled medication was based on the auto-reported medication name.

Allergic rhinitis: Positive answer to "Do you have any nasal allergies, including hay fever?"

Atopic dermatitis/eczema: Positive answer to "Have you ever had eczema or skin allergy?"

Food allergy: Positive answer to "Have you been diagnosed with food allergy by a doctor?"

Environmental tobacco smoke: Positive answer to "Does anyone smoke inside your home?"

Municipality was classified as urban, if the residence was located in a municipality with at least one city (cities must have at least 8000 citizens), according to Portuguese law.

Statistical analysis

The estimates from the sample were weighted to allow for generalization to the target population. A two-stage stratified sampling design was implemented using the complex sampling module of SPSS version 19 (SPSS IBM, New York, NY, USA). First, a simple random sampling method without replacement was used to select a random sample of households with a landline telephone within each stratum (municipality). Second, one eligible household resident within each selected household was randomly selected using a simple random sampling method without replacement. Two types of weights were used. First, weights were used to adjust for the sampling design, taking into account the probability of selection of each subject. Second, poststratification weights were used to adjust for the true gender and age distribution of the target population (weights took into account gender and 5-year age strata in each sampling stratum), thus partially correcting for nonresponse and

noncoverage bias.

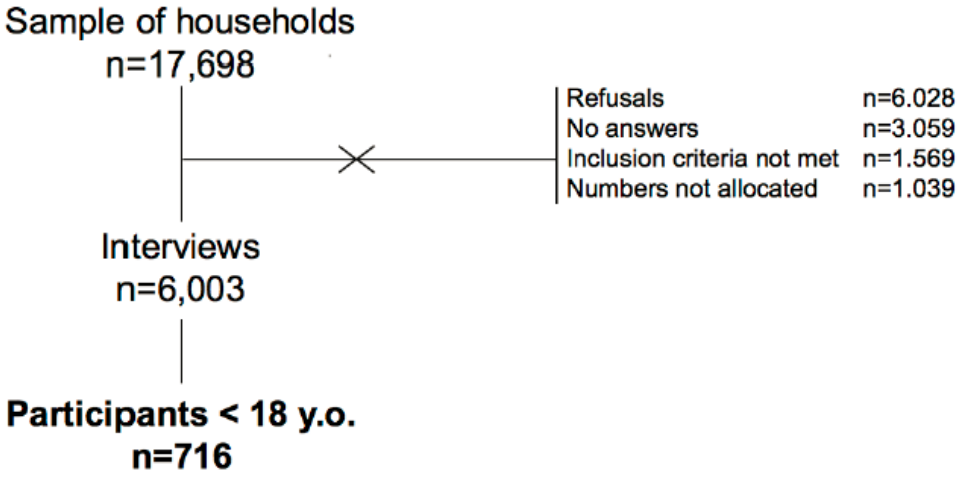

Figure S1. Participants' flow chart.

Table S1. Weighted proportion of asthma-like symptoms and corresponding odds ratio in the Portuguese paediatric population in the previous 12 months according to sociodemographic variables.

	Any symptom[a]			Wheezing			Nocturnal symptoms			Asthma attack	
	% (95%CI)	Odds Ratio[b] (95%CI)		% (95%CI)	Odds Ratio[b] (95%CI)		% (95%CI)	Odds Ratio[b] (95%CI)		% (95%CI)	Odds Ratio[b] (95%CI)
Sex											
Female	36.3 (30.6-42.3)	1.00 (Ref)		14.0 (10.6-18.4)	1.00 (Ref)	p<0.001	32.6 (27.1-38.6)	1.00 (Ref)		3.3 (1.8-5.9)	1.00 (Ref)
Male	42.5 (37.9-47.2)	1.29 (0.94-1.79)		23.9 (20.1-28.2)	**1.96 (1.30-2.97)***		36.8 (32.3-41.6)	1.20 (0.86-1.68)		4.2 (2.7-6.5)	1.29 (0.60-2.78)
Age											
0-5 years	47.9 (40.0-56.0)	1.00 (Ref)		31.0 (24.4-38.5)	1.00 (Ref)		39.8 (32.1-48.1)	1.00 (Ref)		2.8 (1.2-6.8)	1.00 (Ref)
6-12 years	39.2 (33.5-45.2)	0.70 (0.47-1.05)	p=0.005	17.3 (13.7-21.5)	**0.46 (0.30-0.70)***	p<0.001	35.7 (30.3-41.6)	0.84 (0.55-1.28)	p<0.001	5.5 (3.4-8.9)	2.00 (0.71-5.65)
13-17 years	31.8 (26.3-37.8)	**0.51 (0.33-0.77)***		9.9 (7.0-13.9)	**0.24 (0.15-0.40)***		28.8 (23.5-34.7)	**0.61 (0.40-0.94)***		2.5 (1.3-4.6)	0.88 (0.29-2.63)
National region											
North	38.2 (32.3-44.5)	1.00 (Ref)		20.5 (16.0-26.0)	1.00 (Ref)		33.3 (27.7-39.5)	1.00 (Ref)		3.1 (1.7-5.9)	1.00 (Ref)
Center and Islands	43.3 (36.0-50.9)	1.25 (0.84-1.86)		17.4 (13.0-22.9)	0.81 (0.51-1.29)		38.6 (31.6-46.1)	1.26 (0.84-1.89)		2.8 (1.2-6.3)	0.88 (0.30-2.59)
South	37.6 (31.6-44.1)	0.96 (0.66-1.41)		18.8 (14.4-24.0)	0.87 (0.55-1.36)		33.2 (27.3-39.6)	0.99 (0.67-1.46)		5.2 (3.0-8.7)	1.68 (0.71-3.97)
Municipality[d]											
Urban	40.5 (36.1-45.0)	1.00 (Ref)		19.3 (16.1-23.0)	1.00 (Ref)		36.0 (31.7-40.5)	1.00 (Ref)		3.4 (2.1-5.3)	1.00 (Ref)
Rural	35.4 (29.6-41.7)	0.81 (0.58-1.12)		18.0 (14.5-22.2)	0.91 (0.63-1.33)		29.9 (24.3-36.1)	0.76 (0.54-1.07)		5.2 (2.9-9.1)	0.90 (0.53-1.52)
ETS[c]											
No	38.2 (33.7-43.0)	1.00 (Ref)		19.7 (16.3-23.5)	1.00 (Ref)		32.9 (28.5-37.7)	1.00 (Ref)		3.7 (2.2-6.1)	1.00 (Ref)
Yes	41.6 (35.2-48.3)	1.16 (0.82-1.63)		18.0 (13.8-23.2)	0.89 (0.59-1.33)		37.8 (31.7-44.4)	1.24 (0.88-1.75)		3.9 (2.3-6.3)	1.06 (0.50-2.23)

[a]Wheezing *or* waking with breathlessness *or* waking with chest tightness *or* waking with cough *or* asthma attacks. [b]Adjusted odds ratio for gender and age. [c]Municipality was classified as urban if residence was located in municipalities with at least one city (cities must have at least 8000 citizens) according to Portuguese law. [d]Environmental tobacco smoke. *statistically significant

Table S2. Weighted proportion of asthma-like symptoms and corresponding odds ratio in the Portuguese paediatric population in the previous 12 months according to associated comorbidities.

	Any symptom[a]			Wheezing			Nocturnal symptoms			Asthma attack		
	% (95%CI)	Odds ratio[b] (95%CI)		% (95%CI)	Odds Ratio[b] (95%CI)		% (95%CI)	Odds Ratio[b] (95%CI)		% (95%CI)	Odds Ratio[b] (95%CI)	
Rhinitis												
No	34.1 (30.1-38.5)	1.00 (Ref)	p<0.001	14.3 (11.6-17.6)	1.00 (Ref)	p<0.001	29.8 (25.9-34.0)	1.00 (Ref)	p<0.001	2.6 (1.5-4.5)	1.00 (Ref)	p<0.001
Yes	61.2 (53.2-68.7)	**3.67 (2.52-5.39)***		38.5 (31.1-46.4)	**6.01 (3.82-9.46)***		55.1 (47.1-62.8)	**3.36 (2.31-4.89)***		8.3 (5.4-12.7)	**3.48 (1.69-7.17)***	
Eczema												
No	35.0 (30.8-39.5)	1.00 (Ref)	p<0.001	15.0 (12.3-18.3)	1.00 (Ref)	p<0.001	31.0 (26.9-35.3)	1.00 (Ref)	p<0.001	2.0 (1.1-3.6)	1.00 (Ref)	p<0.001
Yes	52.4 (45.6-59.2)	**2.11 (1.51-2.94)***		30.8 (24.8-37.6)	**2.77 (1.85-4.15)***		45.8 (38.9-52.9)	**1.92 (1.36-2.71)***		8.8 (5.6-13.7)	**4.74 (2.16-10.39)***	
Food allergy												
No	39.0 (35.1-42.9)	1.00 (Ref)		18.9 (16.1-22.0)	1.00 (Ref)		34.4 (30.7-38.4)	1.00 (Ref)		3.7 (2.5-5.4)	1.00 (Ref)	
Yes	48.3 (34.4-62.5)	1.58 (0.87-2.88)		22.8 (13.2-36.6)	1.52 (0.78-2.98)		40.0 (27.1-44.5)	1.35 (0.72-2.50)		4.9 (2.1-11.0)	1.35 (0.53-3.44)	

[a]Wheezing *or* waking with breathlessness *or* waking with chest tightness *or* waking with cough *or* asthma attacks. [b]Adjusted odds ratio for gender and age. *Statistically significant

Table S3. Weighted proportion of medication use in children with lifetime asthma or current asthma.

	Lifetime asthma % (95%CI)	Current asthma % (95%CI)
Pulmonary function tests	41.4 (31.6-51.9)	45.7 (33.8-58.1)
Allergy tests (skin and/or blood)	72.1 (62.1-80.3)	79.9 (67.9-88.2)
Any asthma medication at the time of survey[a]	57.5 (47.3-67.1)	76.4 (64.4-85.3)
Inhaled therapy in previous 12 months	48.1 (38.1-58.3)	67.6 (55.5-77.8)
Reliever inhaled meds in previous 12 months	30.2 (21.1-41.1)	40.1 (28.5-53.0)
SABA[b]	23.7 (15.6-34.3)	31.5 (21.0-44.4)
Anticholinergics	6.4 (2.6-15.2)	8.6 (3.4-19.8)
Controller inhaled meds in previous 12 months	31.2 (22.0-42.3)	41.5 (29.6-54.5)
LABA[c]	4.8 (1.6-13.8)	6.4 (2.1-17.9)
Fluticasone	11.2 (5.7-20.9)	14.9 (7.6-27.1)
Budesonide	4.9 (2.1-10.6)	6.5 (2.8-14.0)
Budesonide/Formoterol	5.1 (2.3-11.1)	6.8 (3.0-14.7)
Fluticasone/Salmeterol	9.9 (4.6-19.8)	13.1 (6.2-25.6)
ONLY Reliever inhaled therapy in previous 12 months[d]	17.9 (10.9-27.9)	23.8 (14.6-36.3)

[a]Reference to any asthma medication (inhaled, nebulized, or oral); [b]SABA: short-acting β-agonist; [c]LABA: long-acting β-agonist; [d]Without use of inhaled controller therapy (no specific information on oral control therapy was available)

Output 1b:

Asthma prevalence in Portuguese preschool children: more scientific evidence…

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Authors:	Mário Morais-Almeida, Helena Pité, Ana Margarida Pereira, Manuel Ferreira-Magalhães, João Almeida Fonseca
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Asthma prevalence in Portuguese preschool children: More scientific evidence...

Dear Editor,

We read with interest the research letter ''Asthma prevalence in Portuguese preschool children: The latest scientific evidence'', published by Branco et al.[1] The authors intended to review the major findings of published papers on childhood asthma prevalence in preschool-aged children in our country. We agree with the authors that it is important to have nationwide-based surveys, so we would like to briefly cite two large surveys that were published by our group, and that can contribute to the knowledge about asthma and recurrent wheezing in the same age group (Table 1).[2,3]

The first survey was a cross-sectional, questionnaire-based (ISAAC-adapted) descriptive study of children aged between 3 and 5 years living in mainland Portugal (ARPAkids); a representative sample of 5003 children was included in the analysis.[2]

The prevalence of ''wheezing in the last 12 months'' (current wheezing – CW) was 24.5% [95% confidence interval (CI) 23.3–25.7]; most of these children (61.4%) had had 1–3 wheezing episodes in the previous year, and 7.5% reported >12 episodes. The prevalence of CW was significantly higher in children with a family history of allergic disease; those living in rural regions had a significantly lower wheezing prevalence. No statistically significant differences were found for gender, age, birth weight, or exposure to tobacco smoke at home. The prevalence of ''physician diagnosed asthma'' (PDA) in children with CW was 16.7% [95% CI 14.6–18.8] – vs. 4.6% [95% CI 4.0–5.2] in the general population; it was highest (73.0%) in those with >12 wheezing episodes.

CW was strongly associated with rhinitis in this age group, especially with moderate – severe persistent disease; children with both wheezing and rhinitis had more wheezing episodes and needed treatment more frequently. The number of wheezing episodes significantly increased with increasing rhinitis severity/persistency.[2,4]

The second survey included the paediatric-specific data (n = 716; 21.4% younger than 6 years of age) from a cross-sectional, population-based, all-age, nationwide telephone interview study (INAsma), the first population-based epidemiological study aiming to estimate asthma prevalence in Portugal covering all ages.[3] The methodology of this study has been previously described[5]; a GA2LEN-adapted questionnaire was used. ''Current asthma'' (CA) was defined as a positive answer to ''has the child ever had asthma?'', plus at least one of 4: wheezing, waking with breathlessness or having had an asthma attack in the previous 12 months, or currently taking asthma medication.

In children aged <6 years, the prevalence of CW and CA was 31.0% (95% CI 24.4–38.5) and 6.5% (95% CI 3.5–11.6), respectively. No statistically significant differences were found in the prevalence of CA according to region or other socio-demographic variables, but a higher risk for CA was found in children with rhinitis and eczema.[3]

In conclusion, asthma-like symptoms were present in one-third to one-fourth of Portuguese preschool children; the prevalence of PDA was 4.6% (ARPAkids) and of CA was 6.5% (INAsma). Asthma prevalence can vary significantly according to different study methodologies, namely disease definition, and objective markers of disease must be included in future surveys.

Table 1 Comparison of the main characteristics of the nationwide studies analysis asthma prevalence in pre-school children.

		ARPAKids	INAsma
Study design	Date	February to March 2007	March to May 2010
	Location	Mainland Portugal (nationwide)	Portugal (nationwide)
	Study population	5003 children (aged 3–5 years)	716 children (aged 0–17 years, of which 153 aged < 6 years)
	Aim	To estimate the prevalence of current wheezing in preschoolers; also estimate the prevalence of physician diagnosed asthma	To estimate the prevalence of asthma-like symptoms, current asthma, asthma diagnostic tests and inhaled medication use in children
Methodology	To recruit study population	Stratified random population-based representative sample including all municipalities in mainland Portugal. Kindergartens and parish centers were used for data collection.	Stratified random population-based representative sample including all municipalities in Portugal. Houses with a landline telephone were used for data collection.
	To collect health information	Face-to-face interview with caregivers using an ISAAC-adapted questionnaire	Computer-assisted telephone interview with caregivers using GA2LEN-adapted questionnaire
	Criteria to define asthma	Current wheezing: positive answer to the question ''Has the child had wheezing episodes over the last 12 months?'' Physician-diagnosed asthma: positive answer to the question ''Has a doctor ever said that the child has asthma?''	Current asthma: positive answer to the question ''Has the child ever had asthma?'', plus at least one of 4: wheezing, waking with breathlessness or having an asthma attack in the last 12 months, or currently taking asthma medication.
Outcomes	Asthma prevalence	Current wheezing: 24.5% (95% CI 23.3–25.7) Physician-diagnosed asthma: 4.6% (95% CI 4.0–5.2)	Current asthma in children: 8.4% (95% CI 6.6–10.7) Current asthma in children aged <6 years: 6.5% (95% CI: 3.5–11.6)
	Risk factors	Current wheezing and physician-diagnosed asthma were associated with rhinitis (persistency/severity)/rhinoconjunctivitis and family history of allergic diseases. Physician-diagnosed asthma was associated with the frequency of wheezing episodes. Children living in rural regions had a significantly lower wheezing prevalence (no significant differences were found for physician-diagnosed asthma).	Current asthma was associated with rhinitis and eczema. No statistically significant differences were found in the prevalence of current asthma according to region or other socio-demographic variables.

95% CI–95% confidence intervals.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Prevalence and classification of rhinitis in the elderly: a nationwide survey in Portugal

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Conception and design:	Mário Morais-Almeida
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Data interpretation and manuscript review:	All authors
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ORIGINAL ARTICLE EPIDEMIOLOGY AND GENETICS

Prevalence and classification of rhinitis in the elderly: a nationwide survey in Portugal

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Abstract

Background: Nationwide epidemiologic data on rhinitis in the elderly do not exist. This study aimed to estimate the prevalence of rhinitis in the population aged 65 years or above in mainland Portugal and to characterize and classify rhinitis in this age group.

Methods: Cross-sectional, nationwide, population-based survey of citizens aged 65 years or above, living in mainland Portugal. Current rhinitis (CR) was defined as the presence of at least two symptoms: 'repeated sneezing and itchy nose', 'blocked nose for more than one whole hour', or 'runny nose when not having a cold or flu', either usually or in the last 12 months.

Rhinitis severity was assessed using a visual analogue scale; rhinitis was classified according to ARIA.

Results: Data were obtained from 3678 responders (92.5% response rate). The prevalence of CR was 29.8% (95% confidence interval (CI): 28.4%–31.3%): 49.1% had mild intermittent, 7.0% mild persistent, 27.5% moderate-severe intermittent, and 16.4% moderate-severe persistent rhinitis. Only 38.6% of patients with CR had been physician diagnosed and 38.7% were under treatment for this disease in the previous year. Allergic conjunctivitis symptoms were referred by 68.6% of subjects with CR (rhinoconjunctivitis population prevalence, 20.5% (95% CI: 19.2%–21.8%)).

Conclusions: Rhinitis and rhinoconjunctivitis are common but underdiagnosed and undertreated diseases in the geriatric population. This was the first nationwide epidemiological survey classifying rhinitis according to ARIA guidelines in this age group. More than 40% of old-age patients presented moderate-severe disease.

Allergic rhinitis is a common chronic respiratory disease, with a reported prevalence of up to 40% in children and 30% in adults (1–3). It is frequently associated with allergic conjunctivitis, asthma, and other comorbidities. Considering its high impact on patient's quality of life and economic burden (4, 5), allergic rhinitis is regarded as a global public health problem.

In the last decades, the increase in elderly population has been noteworthy in industrialized countries. The increase in life expectancy goes in parallel with a concern for better quality of life. Studies on allergic and respiratory diseases in geriatric populations have shown that allergens, infections, and irritants can be important triggers of inflammation, regardless of age-related physiologic changes in the immune system, connective tissue, and vasculature that may predispose for chronic rhinitis (6–10).

Whereas rhinitis was considered to be highly prevalent in children and young adults, epidemiologic studies suggest that

it is common in older subjects (11). However, epidemiologic data on rhinitis in the elderly are scarce (12, 13). Studies specifically addressing this age group are needed for a proper analysis of rhinitis in geriatrics, allowing better knowledge and raised awareness concerning this disease in the growing elderly population.

This study aimed to estimate the prevalence of rhinitis in the population aged 65 years or above in mainland Portugal and to classify its severity according to ARIA. Secondary aims were to characterize rhinitis symptoms, associated conjunctivitis, and asthma, and to describe the frequency of physician diagnosis and rhinitis medication use.

Methods

Study design and participants

Population-based, cross-sectional survey applied by face-to-face interview to citizens aged 65 years or above, living in mainland Portugal.

A stratified (by gender, age, and region), multistep defined sample was used. In a first phase, the number of needed participants from each Portuguese region was defined. This estimation was based on the data from the 2001 Portuguese Census on individuals aged 65 years or above (14) and took into account the second level of the Portuguese Nomenclature of Territorial Units for Statistics (NUTS II), which divides mainland Portugal in five regions: North, Centre, Lisbon and Tagus Valley, Alentejo, and Algarve (Figure S1 represents the distribution of the included elders in each region across mainland Portugal). In a second phase, municipalities were selected within each of the five Portuguese regions until reaching a prespecified number of elder subjects in proportion to the sample size. The selection criteria of municipalities correspond to the aging index, population density, and purchasing power, indicators that summarize the main regional social disparities (14). The distribution by municipalities was based on the levels of differentiation in each region against the factors used to stratify the sample. Population density and purchasing power levels were defined considering the median (national) and standard deviation. The final selection was made using a random-route methodology, where households and nursing homes were included, in each location. (Table S1 shows the comparison between the Portuguese population aged 65 years or above, the predicted sample, and the true sample, considering age, gender, and region).

Informed consent was obtained from all participants. This study's protocol was reviewed and approved by the Ethics Committee of the Hospital CUF-Descobertas, Lisbon, Portugal.

Instruments and data collection

The instrument for data collection (Table S2) was adapted from the questionnaire used in our previous study (3). A few questions were added regarding age of onset of rhinitis symptoms, physician-diagnosed asthma, and use of medication for asthma treatment. The questionnaire included items previously used in the European Community Respiratory Health Survey (15) (ECRHS) and was adapted to old-age subjects.

Rhinitis severity was assessed using a 0–10 visual analogue scale (VAS), as previously suggested (16).

Questionnaires were applied by trained interviewers between May and July 2008.

Definitions

Definitions used in this study are presented in Table 1. In individuals with rhinitis, it was further classified into intermittent/persistent and mild/moderate-severe, according to current ARIA guidelines (4) (Table 1).

Sample size and statistical analysis

To estimate the prevalence of rhinitis in the elderly Portuguese population, with an expected national prevalence of 25% and considering an error margin of 1.5% and a confidence interval of 95%, we would need a sample including 3500 subjects. A nonresponse rate of 12.5% was assumed. Therefore, 4000 subjects were invited to participate.

Categorical variables were described using absolute and relative frequencies with 95% confidence intervals (95% CI); comparisons were performed using the Pearson's chi-squared test.

Continuous variables were described with mean and standard deviation (SD); for comparisons, an independent samples t-test was used.

A significance level of 5% was considered.

Multiple logistic regression analysis was used to calculate odds ratios (ORs) with 95% CI separately for current rhinitis, physician-diagnosed rhinitis, and rhinoconjunctivitis (detailed in Table S3).

Data analyses were performed using SPSS® version 17.0 for Windows (IBM SPSS, Chicago, IL, USA).

Results

Participants

A total of 3699 of 4000 invited subjects answered the questionnaire (response rate 92.5%). There was no evidence of different response rates according to age. From the answered questionnaires, 21 were excluded: 13 subjects were aged <65 years and eight had insufficient information due to incomplete interviews. A total of 3678 individuals were included in the analysis (Table S4 – shows the missing data regarding each variable). Participants' socio-demographic characteristics are summarized in Table 2.

Prevalence of current rhinitis, physician-diagnosed rhinitis, and rhinoconjunctivitis

The prevalence of current rhinitis was 29.8% (95% CI: 28.4%–31.3%). Current rhinitis prevalence was similar in men and women as well as in age groups, and no differences

Table 1 Definitions used in this study

Variable	Source	Definition
Current rhinitis	Based on ECRHS and ARIA (3, 4,15)	Presence, usually or in the last 12 months, of at least two of the following symptoms: 'repeated sneezing and itchy nose'; 'blocked nose for more than one whole hour'; or 'runny nose when not having a cold or flu'
Intermittent rhinitis	Built according to ARIA (4)	Current rhinitis with nasal symptoms lasting <4 days in a week or lasting more than 4 days/week, but <4 consecutive weeks
Persistent rhinitis	Built according to ARIA (4)	Current rhinitis with nasal symptoms lasting for at least 4 days in a week and for more than 4 consecutive weeks
Mild rhinitis	Adapted from Bousquet et al. (16)	Current rhinitis that had a visual analogue scale severity score ranging between 0 and 5
Moderate-severe rhinitis	Adapted from Bousquet et al. (16)	Current rhinitis that had a visual analogue scale severity score ranging between 6 and 10
Rhinoconjunctivitis	Adapted from ECRHS (15)	Presence of rhinitis and a positive answer to the question 'Do nasal symptoms usually occur along with red or itchy–watery eyes?'
Physician-diagnosed rhinitis	Additional question (3)	Positive answer to the question 'Has a doctor ever said you have rhinitis?'
Previous performed skin prick tests	Additional question (3)	Positive answer to the question 'Has a doctor ever asked you to make skin prick tests?'
Physician-diagnosed asthma	Adapted from ECRHS (15)	Positive answer to the question 'Has a doctor ever said you have asthma?'
Current treatment for rhinitis	Adapted from ECRHS (15)	Positive answer to the question 'Have you had any medication for rhinitis (nasal topical drug or pill) in the last 12 months?'
Current treatment for asthma	Adapted from ECRHS (15)	Positive answer to the question 'Do you take medication for asthma?'

Table 2 Socio-demographic characteristics of the participants ($n = 3678$)

	n (%)
Gender	
Female	2151 (58.5)
Age, years	
65–74	2128 (57.9)
75–84	1204 (32.7)
≥85	346 (9.4)
Residency	
Own house	2657 (73.2)
Relatives	632 (17.4)
Nursing home	343 (9.4)
Municipality	
Urban	2752 (76.5)
Rural	845 (23.5)
Portuguese Region	
North	1168 (31.8)
Centre	789 (21.5)
Lisbon and Tagus Valley	1271 (34.6)
Alentejo	284 (7.7)
Algarve	166 (4.5)

were observed between urban and rural municipalities (Table 3). In two Portuguese regions, current rhinitis prevalence was higher than the national prevalence ($P < 0.0001$): Alentejo (57.7%) and Lisbon and Tagus Valley (35.7%). In the Algarve (8.4%) and Centre (14.6%), current rhinitis prevalence was significantly lower than the national prevalence ($P < 0.001$). Adjusting for independent variables in Table S3, living in Alentejo yielded a high OR, 3.19 (95% CI: 2.45–4.17), for current rhinitis, while living in Lisbon and Tagus Valley region was a weak but statistically significant risk for current rhinitis (OR 1.29; 95% CI: 1.08–1.52). Living in the Centre or in the Algarve was a protective factor for current rhinitis (Table S3).

Physician-diagnosed rhinitis was reported by 38.6% subjects with current rhinitis (population prevalence 13.1%, 95% CI: 12.0%–14.2%) and 38.7% had been under treatment for this disease in the previous year. The frequency of current rhinitis among those with physician-diagnosed rhinitis was 87.9%. The prevalence of physician-diagnosed rhinitis was lower than that of current rhinitis in all Portuguese regions (Table 3). This difference was greater in Alentejo, a region associated with a high risk of current rhinitis, but a low risk for physician-diagnosed rhinitis (Table S3).

Symptoms of allergic conjunctivitis were reported by 68.6% of subjects with current rhinitis, accounting for a prevalence of rhinoconjunctivitis of 20.5% (95% CI: 19.2%–21.8%) in the studied population (Table 3). The risk of rhinoconjunctivitis was dependent on the Portuguese region. Living in a nursing home was significantly associated with current rhinitis, physician-diagnosed rhinitis, and rhinoconjunctivitis (Table S3).

Elders with physician-diagnosed asthma or currently on treatment for asthma presented significantly higher prevalence of current rhinitis, physician-diagnosed rhinitis, and rhinoconjunctivitis (Table 3). In univariate analysis, having physician-diagnosed asthma was strongly associated with all three conditions, in particular with current rhinitis and rhinoconjunctivitis (Table S3).

Table 3 Prevalence of current rhinitis, physician-diagnosed rhinitis, and rhinoconjunctivitis (n = 3678)

	Current rhinitis % [95% CI]	Physician-diagnosed rhinitis % [95% CI]	Rhinoconjunctivitis % [95% CI]
Whole sample	29.8 [28.4–31.3]	13.1 [12.0–14.2]	20.5 [19.2–21.8]
Gender	0.086*	0.052*	0.087*
Male	28.3 [26.0–30.6]	11.8 [10.2–13.4]	19.1 [17.1–20.1]
Female	30.9 [29.0–32.9]	14.0 [12.5–15.5]	21.4 [19.7–23.1]
Age, years	0.667*	0.530*	0.378*
65–74	30.2 [28.3–32.2]	12.6 [11.2–14.0]	20.9 [19.2–22.6]
75–84	28.9 [26.3–31.5]	14.0 [12.0–16.0]	19.3 [17.1–21.5]
≥85	30.9 [26.0–35.8]	13.1 [9.5–16.7]	22.3 [17.9–26.7]
Municipality	0.082*	0.207*	0.419*
Urban	30.7 [29.0–32.4]	13.5 [12.2–14.8]	20.9 [19.4–22.4]
Rural	27.6 [24.6–30.6]	11.9 [9.8–14.0]	19.7 [17.0–22.4]
Region	<0.0001*	<0.0001*	<0.0001*
North	30.0 [27.4–32.6]	16.4 [14.3–18.5]	23.2 [20.8–25.6]
Centre	14.6 [12.1–17.1]	9.5 [7.5–11.6]	8.7 [6.7–10.7]
Lisbon and Tagus Valley	35.7 [33.1–38.3]	15.0 [13.0–17.0]	24.4 [22.0–26.8]
Alentejo	57.7 [52.0–63.5]	5.3 [2.7–7.9]	33.8 [28.3–39.3]
Algarve	8.4 [4.2–12.6]	4.8 [1.5–8.1]	4.2 [1.2–7.3]
Physician-diagnosed asthma	<0.0001*	<0.0001*	<0.0001*
Absent	23.6 [22.2–25.1]	9.7 [8.7–10.7]	14.6 [13.4–15.8]
Present	81.1 [77.3–84.9]	40.9 [36.1–45.7]	68.3 [63.8–72.9]
Current treatment for asthma	<0.0001*	<0.0001*	<0.0001*
Absent	25.7 [24.2–27.2]	12.2 [11.1–13.3]	16.3 [15.1–17.5]
Present	86.6 [82.6–90.6]	39.8 [34.1–45.5]	71.7 [66.4–77.0]

*P-value.

Rhinitis characterization and classification

Among participants classified as having current rhinitis, the most commonly reported symptom was 'runny nose when not having a cold or flu' (31.1%), followed by 'repeated sneezing and itchy nose' (30.0%; Fig. 1); 22.5% reported to have all the questioned symptoms either usually or in the last 12 months.

In 39.0% of these individuals, rhinitis symptoms had started between 20 and 40 years of age; 19.6% reported that rhinitis symptoms started before the age of 20, 16.5% between 40 and 60 years, and 4.6% after the age of 60 (20.3% did not know/did not answer). The overall symptom duration was of more than 25 years in 60.1% of the elders. There were no significant gender differences in the age of onset of rhinitis symptoms.

Nasal symptoms were present for more than 4 days per week in 42.8% of cases and more than 4 weeks in a year in 50.8%. The mean (SD) value for rhinitis severity, considering the 0–10 VAS, was 4.9(2.3). The overall VAS results are presented in Fig. 2.

Rhinitis was classified as mild intermittent in 49.1% of all cases, moderate-severe intermittent in 27.5%, mild persistent in 7.0%, and moderate-severe persistent in 16.4%. The prevalence of each rhinitis type and its characterization are expressed in Table 4. Elderly subjects living in urban regions had a higher prevalence of moderate-severe intermittent and mild persistent rhinitis, while those living in rural regions had higher frequency of moderate-severe persistent rhinitis (Table 4). Two Portuguese regions, Alentejo and Lisbon and Tagus Valley, presented higher prevalence of intermittent

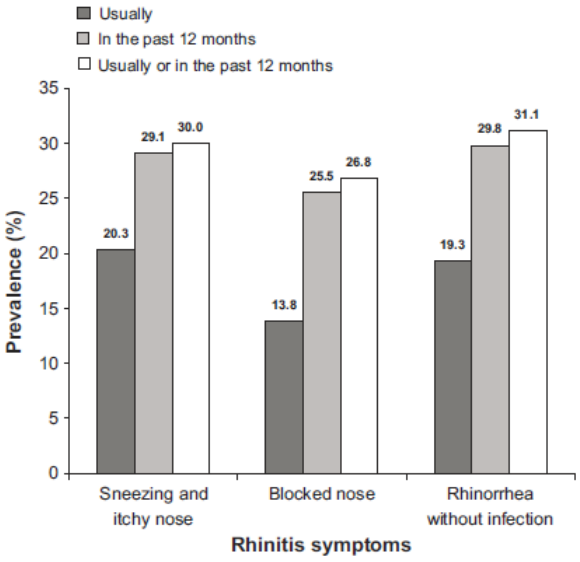

Figure 1 Prevalence of rhinitis symptoms reported either usually or in the past 12 months (*n* = 3678).

rhinitis, compared with the other regions (57.0% *vs* 19.6% and 30.1% *vs* 18.4%, respectively, $P < 0.0001$). Those elders living in the North had higher prevalence of persistent rhinitis (11.9% *vs* 4.5%, $P < 0.0001$), and elders living in the Algarve presented lower prevalence of all classes of rhinitis (Table 4).

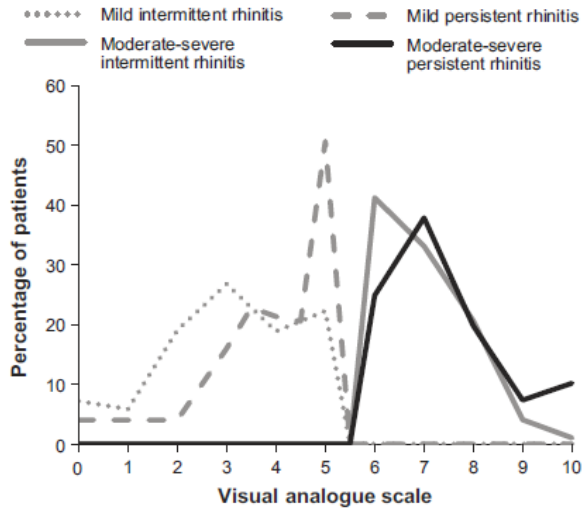

Figure 2 Distribution of patients according to visual analogue scale results in each ARIA class.

Individuals with physician-diagnosed asthma and those on current treatment for asthma, when compared with those without, presented higher prevalence of all classes of rhinitis, including mild intermittent (Table 4).

Physician-diagnosed rhinitis and previously performed skin prick tests were more frequent in elderly people with moderate-severe rhinitis (57.4% *vs* 23.6% and 70.2% *vs* 50.4% in mild rhinitis, respectively, $P < 0.0001$) and in those with persistent rhinitis (80.2% *vs* 25.5% and 75.4% *vs* 54.1% in intermittent rhinitis, respectively, $P < 0.0001$). Likewise, rhinitis treatment in the previous year was more frequent in elderly people with moderate-severe rhinitis (56.9% *vs* 24.1% in mild rhinitis, $P < 0.0001$) and in those with persistent rhinitis (80.2% *vs* 25.8% in intermittent rhinitis, $P < 0.0001$).

Rhinoconjunctivitis was also more frequent in subjects with moderate-severe (83.7% *vs* 57.0% in mild rhinitis, $P < 0.0001$) and persistent rhinitis (92.0% *vs* 61.6% in intermittent rhinitis, $P < 0.0001$).

Discussion

In the Portuguese population aged 65 years or above, we found a high prevalence of current rhinitis and of rhinoconjunctivitis – 29.8% and 20.5%, respectively. Furthermore, this study showed that these diseases were underdiagnosed and undertreated in this age group, despite a overall symptom duration of more than 25 years in the majority of subjects and that almost half had moderate-severe disease.

Table 4 Prevalence of rhinitis according to classification categories ($n = 3678$)

	Mild intermittent % [95% CI]	Moderate-severe intermittent % [95% CI]	Mild persistent % [95% CI]	Moderate-severe persistent % [95% CI]
Whole sample	14.4 [13.3–15.5]	8.1 [7.2–9.0]	2.0 [1.6–2.5]	4.8 [4.1–5.5]
Gender	0.846*	0.113*	0.788*	0.483*
Male	14.3 [12.5–16.1]	7.2 [5.9–8.5]	2.0 [1.3–2.7]	4.5 [3.5–5.5]
Female	14.5 [13.0–16.0]	8.6 [7.4–9.8]	2.1 [1.5–2.7]	5.0 [4.1–5.9]
Age, years	0.155*	0.041*	0.936*	0.835*
65–74	15.3 [13.8–16.8]	7.7 [6.6–8.8]	2.0 [1.4–2.6]	4.7 [3.8–5.6]
75–84	13.6 [11.7–15.5]	7.7 [6.2–9.2]	2.2 [1.4–3.0]	5.1 [3.9–6.3]
≥85	11.8 [8.4–15.2]	11.6 [8.2–15.0]	2.0 [0.5–3.5]	4.3 [2.2–6.4]
Municipality	0.837*	<0.0001*	0.004*	<0.0001*
Urban	14.4 [13.1–15.7]	9.4 [8.3–10.5]	2.4 [1.8–3.0]	4.1 [3.4–4.8]
Rural	14.7 [12.3–17.1]	3.9 [2.6–5.2]	0.8 [0.2–1.4]	7.3 [5.6–9.1]
Region	<0.0001*	<0.0001*	0.017*	<0.0001*
North	12.4 [10.5–14.3]	4.6 [3.4–5.8]	3.1 [2.1–4.1]	8.8 [7.2–10.4]
Centre	6.8 [5.0–8.6]	2.2 [1.2–3.2]	2.0 [1.0–3.0]	3.2 [2.0–4.4]
Lisbon and Tagus Valley	17.0 [14.9–19.1]	13.1 [11.3–15.0]	1.6 [0.9–2.3]	3.8 [2.8–4.9]
Alentejo	37.7 [32.1–43.3]	19.4 [14.8–24.0]	0.7 [0.1–2.5]	0.0 [0.0–1.1]
Algarve	4.8 [1.6–8.1]	1.8 [0.7–3.7]	0.6 [0.0–3.3]	0.0 [0.0–1.8]
Physician-diagnosed asthma	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Absent	12.9 [11.8–14.1]	6.3 [5.5–7.1]	1.5 [1.1–1.9]	2.4 [1.9–2.9]
Present	26.7 [22.4–31.0]	22.7 [18.6–26.8]	6.2 [3.8–8.6]	24.2 [20.0–28.4]
Current treatment for asthma	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Absent	13.6 [12.5–14.8]	6.7 [5.9–7.5]	1.7 [1.3–2.1]	3.3 [2.7–3.9]
Present	24.7 [19.6–29.8]	24.7 [19.6–29.8]	6.5 [3.6–9.4]	23.7 [18.7–28.7]

* *P*-value.

The frequency of rhinitis in the elderly has been addressed in very few elderly-targeted studies. Ventura et al. (17) found that rhinitis was present in 16.8% of the patients aged over 65 years referred to an allergology unit. Population-based studies are even scarcer. Only one study performed in Mexico City has shown that allergic rhinitis was present in 3.6% of 333 elderly subjects who were attending a social security recreative center (12). In general epidemiologic studies, subgroup analysis reported rhinitis prevalence between 16.1% and 25.9% in the elderly (3, 18–20). However, different selection criteria and rhinitis definitions restrict interstudy comparisons.

Although limited by the lack of physician diagnosis confirmation, our study is truly population based, nationwide, and targeted to this specific age group. The sampling strategy allowed that the number of individuals of each gender, age, and Portuguese region is reliably correspondent to the structure of the resident population in mainland Portugal (aged 65 years or above), according to the data from the 2001 National Census (14). Moreover, this is the first large population-based study reporting rhinitis classification according to ARIA guidelines in this age group.

The definition of current rhinitis used in the present study, combining at least two nasal symptoms, is suggestive of allergic rhinitis (4). This was further reinforced as there were associated symptoms of red, itchy eyes and tearing (4), reported by the majority of our cases. However, nonallergic conditions may cause similar symptoms, such as irritants, atrophic, drug-induced, or infectious rhinitis (4). Therefore, allergic rhinitis was not defined, as this was only a questionnaire-based survey, not including any demonstration of specific immunopathologic mechanisms. Still, this definition of rhinitis was important to exclude some of other causes, such as the so-called rhinitis of the elderly, characterized by clear rhinorrhea without other nasal symptoms (4).

Our present findings are in agreement with our previous study reporting rhinitis prevalence in adults in Portugal (3). That study, using questions and a rhinitis definition that were similar to those used in the present report, found high rhinitis prevalence in adults aged 16–95 years (26.1%). In the same study (3), a subpopulation analysis found that rhinitis prevalence in subjects aged above 65 years was 25.9%. The higher prevalence observed in the present study (29.8%) may reflect either a more accurate estimation of rhinitis in the elderly or an actual increasing tendency in rhinitis prevalence in this age group over the last years. A recent nationwide epidemiologic study performed in 2010 in Portugal, including individuals from all age groups and reporting rhinitis prevalence as a secondary aim, found that 22.1% of included subjects had rhinitis (21). However, direct comparisons are limited because different sampling and definitions for rhinitis were used.

Furthermore, we found a higher prevalence of rhinoconjunctivitis than that previously reported in adults (18.4%) (3); this finding is in agreement with a recent study, suggesting that elderly patients have rhinitis plus conjunctivitis more frequently than young adults (22).

The most common nasal symptom in elders with current rhinitis was watery rhinorrhea, which differs from studies in allergic rhinitis in adults (3), where sneezing and itchy nose were the most reported symptoms. This may be due to concomitant age-related changes in nasal physiology in the elderly (nasal glandular atrophy, vascular changes, decreased nasal humidification, decreased mucociliary clearance, and structural changes of the nose) (7, 8, 10, 23), which may contribute to more frequent rhinorrhea. In fact, it is likely that several mechanisms underlie the pathogenesis of rhinitis in the elderly, with potential interaction between inflammatory conditions and the influence of aging on nasal physiology (24).

As defined by ARIA, we found that 49.1% of the elders had mild intermittent rhinitis, 7.0% mild persistent rhinitis, 27.5% moderate-severe intermittent rhinitis, and 16.4% moderate-severe persistent rhinitis. Cross-sectional and longitudinal studies have shown that rhinitis symptoms become milder with age (24–26). However, in this study, more than 40% of the elders with rhinitis presented moderate-severe disease.

Our data emphasize a significant low prevalence of physician diagnosis, previous diagnostic skin prick tests, and treatment for rhinitis. This is especially relevant in the considerable group of older adults reporting moderate-severe symptoms who lack physician diagnosis and treatment. It was interesting to notice that, after adjusting for independent variables, living in nursing homes was associated with current rhinitis, rhinoconjunctivitis, and also with physician-diagnosed rhinitis. Despite more symptomatic, living in a nursing home may foster better access to health care.

We also found regional differences across mainland Portugal. Living in Alentejo or in Lisbon and Tagus Valley region was a significant risk of current rhinitis. The greater difference between current rhinitis and physician-diagnosed rhinitis prevalence was observed in Alentejo. This may be related to the fact that the vast majority of elders living in this region had intermittent rhinitis (98.8%), but may also reflect lower access to health care in this area. Alentejo is an inland region, also characterized by the highest absolute pollen concentrations in mainland Portugal, which occur seasonally, due fundamentally to the pollination of Poaceae (27). This may be associated with the high frequency of intermittent rhinitis we observed in this region.

Although overall rhinitis prevalence did not differ between elders living in rural or urban regions, those living in a rural region had a higher prevalence of moderate-severe persistent rhinitis. This was an unexpected result. Several studies have found that the prevalence of allergic rhinitis is higher in urban than in rural areas (28–30). Moreover, in our previous study in adults (3), rural inhabitants presented significantly higher levels of intermittent rhinitis. It is possible that confounding factors, such as lifetime changes in the living region (from urban to rural regions and vice versa), may contribute to these results.

The importance of the underdiagnosis of rhinitis is further reinforced by the association we observed between all ARIA classes of rhinitis and asthma in this age group. Current rhinitis and rhinoconjunctivitis were strongly associated with

physician-diagnosed asthma. Although only self-reported, physician-diagnosed asthma was studied, our results support an extensive nose–lung interaction in older individuals, as observed by others (9, 31–33). This association may justify the importance of rhinitis physician diagnosis, including in milder forms of the disease.

Addressing rhinitis in the geriatric population is recognized as an important existing unmet need (4), especially considering the increase in the elderly population throughout Europe together with the aim to stimulate active, healthy aging. The present study reveals that rhinitis and rhinoconjunctivitis are highly prevalent but underdiagnosed and undertreated diseases in the elderly population, despite that over 40% have moderate-severe disease.

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Author contributions

HP participated in data analysis and interpretation and wrote the manuscript draft, AMP participated in data interpretation and review, ATB and CN participated in the study conception, JB provided critical review during the project, JAF participated in data analysis and interpretation and provided critical review during the project, and MMA coordinated the study participating in all stages and tasks. All authors have reviewed and approved the final manuscript.

Conflicts of interest

Mário Morais-Almeida (MMA) received travel grants, speaking fees, and honoraria for advisory boards from Astrazeneca, GSK, FAES Farma, MSD, Novartis, OM Pharma, Pfizer, Sanofi, Schering-Plough, Siemens Diagnostics. Ana Todo-Bom (ATB) received travel grants, speaking fees, and honoraria for advisory boards from GSK, FAES Farma, MSD, Novartis. Jean Bousquet (JB) received honoraria for scientific and advisory boards, lectures during meetings, press conferences from Stallergènes, Actelion, Almirall, AstraZeneca, Chiesi, GSK, Merck, MSD, Novartis, OM Pharma, Sanofi-Aventis, Schering-Plough, Teva, Uriach. João Almeida Fonseca (JAF) has received an unrestricted research grant from Aerocrine AB and honoraria for travel grants, speaking fees, and advisory boards from Astrazeneca, GSK, MSD, and Novartis. Helena Pité (HP), Ana Margarida Pereira (AMP), and Carlos Nunes (CN) have no conflicts of interest to declare.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Comparison between the Portuguese population aged 65 years or above, the predicted sample, and the true sample, considering age, gender, and region.

Table S2. Study's questionnaire.

Table S3. Univariate (unadjusted) and multivariate (adjusted) analysis for risk of 'current rhinitis', 'physician-diagnosed rhinitis', or 'rhinoconjunctivitis'.

Table S4. Missing data.

Figure S1. Distribution of the included elders in each region across mainland Portugal.

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Supporting Information

Table S1: Comparison between the Portuguese population aged 65 years or above, the predicted sample and the true sample, considering age, gender and region

	Portuguese population (Census 2001; n=1628596)		Predicted sample (n=3500)		True sample (n=3678)		Difference from the predicted*
	n	%	n	%	n	%	
North	514758	31.61	1106	31.61	1168	31.76	0.15
65-74 years	306679	18.83	659	18.83	688	18.71	-0.12
75-84 years	163994	10.07	352	10.07	369	10.03	-0.04
85-94 years	42108	2.58	90	2.58	107	2.91	0.33
≥ 95 years	1977	0.12	4	0.12	4	0.11	-0.01
Female	301928	18.54	649	18.54	686	18.65	0.11
Center	348734	21.41	749	21.41	789	21.45	0.04
65-74 years	196360	12.06	422	12.06	441	11.99	-0.07
75-84 years	118003	7.25	254	7.25	270	7.34	0.09
85-94 years	32770	2.01	70	2.01	74	2.01	0.00
≥ 95 years	1601	0.10	4	0.10	4	0.11	0.01
Female	200862	12.33	432	12.33	459	12.48	0.15
Lisbon and Tagus valley	565617	34.73	1216	34.73	1271	34.56	-0.17
65-74 years	337646	20.73	726	20.73	745	20.26	-0.47
75-84 years	179868	11.04	386	11.04	413	11.23	0.19
85-94 years	45739	2.81	98	2.81	107	2.91	0.10
≥ 95 years	2364	0.16	6	0.16	6	0.16	0.00
Female	332785	20.43	715	20.43	759	20.64	0.21
Alentejo	125874	7.73	271	7.73	284	7.72	-0.01
65-74 years	71605	4.40	154	4.40	161	4.38	-0.02
75-84 years	42225	2.59	91	2.59	96	2.61	0.02
85-94 years	11488	0.71	25	0.71	26	0.71	0.00
≥ 95 years	556	0.03	1	0.03	1	0.03	0.00
Female	69704	4.28	150	4.28	157	4.27	-0.01
Algarve	73613	4.52	158	4.52	166	4.51	-0.01
65-74 years	41268	2.53	89	2.53	93	2.53	0.00
75-84 years	24943	1.53	54	1.53	56	1.52	-0.01
85-94 years	7067	0.43	15	0.43	16	0.44	0.01
≥ 95 years	335	0.02	1	0.02	1	0.03	0.01
Female	40636	2.50	88	2.50	90	2.45	-0.05

*Difference: % true sample - % predicted sample

Table S2: Study's questionnaire

ID number_____

Interviewer_____

Region_____

I – Demographic data

1. Gender: Male ___ Female ___ 2. Age: ___

3. Residency: Nursing home _____ Own house _____ Relatives _____

4. Municipality of residence _____ 5. Living region: urban ___ rural___

II – Rhinitis symptoms

6. Do you **usually** have:

a) repeated sneezing and itchy nose?..... Yes ___ No___

b) blocked nose for more than one whole hour?..... Yes ___ No___

c) runny nose when you do not have a cold or flu?..... Yes ___ No___

7. **In the past 12 months** have you had:

a) repeated sneezing and itchy nose?..... Yes ___ No___

b) blocked nose for more than one whole hour?..... Yes ___ No___

c) runny nose when you do not have a cold or flu?..... Yes ___ No___

III – Clinical and therapeutic data - If you have answered YES to at least one of the previous questions:

8. At what age did these symptoms start?

Doesn't know/Didn't answer ___ Before 20 years-old ___ 20 to 40 years-old ___ 40 to 60 years-old ___ After 60 years-old ___

9. Do nasal symptoms usually occur along with red or itchy-watery eyes?..... Yes ___ No___

10. Has this nose problem occurred for more than 4 days in a week?..... Yes ___ No___

11. Has this nose problem occurred for more than 4 consecutive weeks in a year?..... Yes ___ No___

12. In a 0 (min.) to 10 (max.) scale, how would you classify the severity of your nose problem?..... 0_____10
(ask the respondent to point the most appropriate score in the attached visual analogue scale)

13. Has a doctor ever said you have rhinitis?..... Yes ___ No___

14. Have you had any medication for rhinitis (nasal topic drug or pill) in the past 12 months?..... Yes ___ No___

15. Has a doctor ever said you have asthma?..... Yes ___ No___

15.1 If yes, do you take medication for asthma?..... Yes ___ No___

16. Has a doctor ever asked you to make skin prick tests?..... Yes ___ No___

Multiple logistic regression analysis

Univariate logistic regression models were developed using independent variables as risk factors for "current rhinitis", "physician-diagnosed rhinitis" or "rhinoconjunctivitis". The univariate models included both possible risk factors (gender, age group, Portuguese region and residency) and "physician-diagnosed asthma" as a possible predictive factor (frequently associated pathology that may share common risk factors). Multivariate logistic regression models were developed for "current rhinitis", "physician-diagnosed rhinitis" or "rhinoconjunctivitis". Only possible risk factors were included in the multivariate logistic regression model; they were chosen according to the p-value of the univariate analysis, considering a cut-off of <0.250. The Hosmer-Lemeshow statistics was used to assess calibration of the multivariate models; a p>0.05 was deemed necessary to consider that the model was calibrated. Results of both univariate and multivariate logistic regression models were presented as odds ratio (OR) with [95% confidence interval (CI)].

Table S3: Univariate (unadjusted) and multivariate (adjusted) analysis for risk of "current rhinitis", "physician-diagnosed rhinitis" or "rhinoconjunctivitis" (multivariate analysis included only possible risk factors). Statistically significant results are presented in bold.

	Current rhinitis		**Physician-diagnosed rhinitis**		**Rhinoconjunctivitis**	
	Unadjusted	**Adjusted (n=3632)**	**Unadjusted**	**Adjusted (n=3629)**	**Unadjusted**	**Adjusted (n=3626)**
	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)
Gender, male	0.88 (0.76-1.02)	0.89 (0.76-1.03)	0.82 (0.68-1.00)	0.85 (0.70-1.04)	0.87 (0.74-1.02)	0.89 (0.75-1.05)
Age group, *years*	*0.667**	NI	*0.530**	NI	*0.375**	NI
65-74 years	1		1		1	
75-84 years	0.94 (0.81-1.10)		1.13 (0.92-1.39)		0.91 (0.76-1.08)	
≥85 years	1.04 (0.81-1.33)		1.05 (0.75-1.47)		1.09 (0.83-1.43)	
Portuguese region	***<0.001****	***<0.001****	***<0.001****	***<0.001****	***<0.001****	***<0.001****

North	1		1		1		1		1		1	
Center	**0.40**	**(0.32-0.50)**	**0.41**	**(0.33-0.52)**	**0.54**	**(0.40-0.71)**	**0.55**	**(0.41-0.73)**	**0.32**	**(0.24-0.42)**	**0.33**	**(0.25-0.43)**
Lisbon and Tagus Valley	**1.30**	**(1.10-1.54)**	**1.29**	**(1.08-1.52)**	0.90	(0.72-1.12)	0.88	(0.71-1.10)	1.06	(0.88-1.28)	1.04	(0.86-1.26)
Alentejo	**3.19**	**(2.45-4.17)**	**3.31**	**(2.53-4.33)**	**0.28**	**(0.17-0.49)**	**0.29**	**(0.17-0.51)**	**1.68**	**(1.27-2.22)**	**1.72**	**(1.30-2.28)**
Algarve	**0.22**	**(0.12-0.38)**	**0.25**	**(0.14-0.44)**	**0.26**	**(0.13-0.54)**	**0.20**	**(0.08-0.49)**	**0.15**	**(0.07-0.31)**	**0.15**	**(0.07-0.35)**
Residency	***<0.001****		***0.006****		***<0.001****		***0.009****		***<0.001****		***0.001****	
Own home	1		1		1		1		1		1	
With relatives	**1.31**	**(1.09-1.57)**	1.17	(0.97-1.42)	**1.36**	**(1.06-1.74)**	1.27	(0.99-1.63)	**1.54**	**(1.25-1.88)**	**1.38**	**(1.12-1.70)**
Nursing home	**1.47**	**(1.17-1.86)**	**1.45**	**(1.14-1.84)**	**1.75**	**(1.30-2.35)**	**1.52**	**(1.13-2.05)**	**1.53**	**(1.18-1.98)**	**1.43**	**(1.10-1.87)**
Physician-diagnosed asthma	**13.86**	**(10.66-18.02)**	NI		**6.46**	**(5.13-8.13)**	NI		**22.15**	**(17.53-27.98)**	NI	

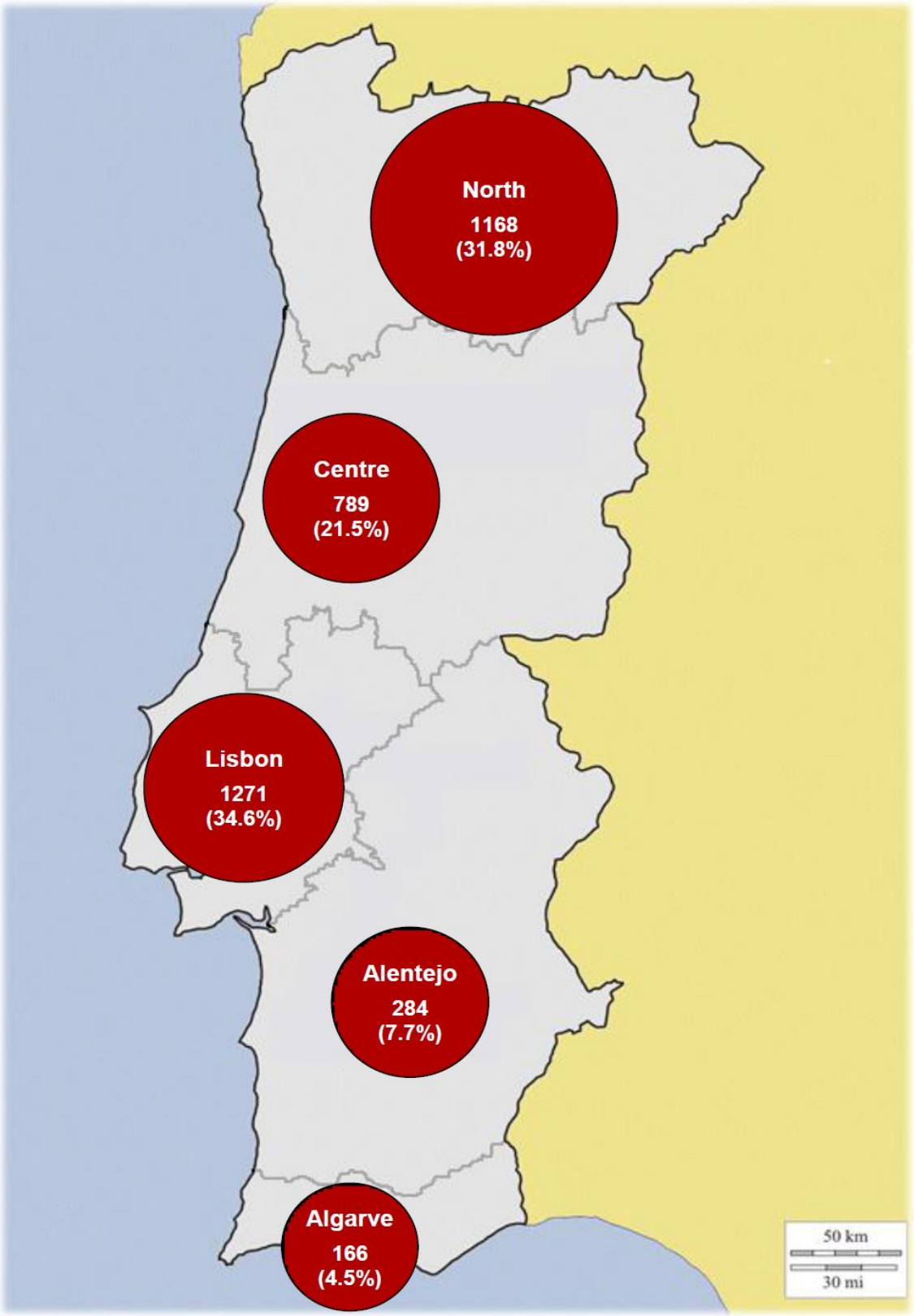
* p-value ; OR, odds ratio; 95%CI, 95% confidence interval; NI , Not included

Table S4: Missing data

	n*	(% of total participants)
Gender	0	(0.0)
Age	0	(0.0)
Municipality	81	(2.2)
Residency	46	(1.3)
Portuguese region	0	(0.0)
Current rhinitis	0	(0.0)
Rhinitis classification		
Intermittent/persistent	6	(0.2)
Mild/Moderate-severe	18	(0.5)
Physician-diagnosed rhinitis	3	(0.1)
Age of onset of rhinitis symptoms	5	(0.5)
Rhinitis treatment	0	(0.0)
Previous skin prick tests	0	(0.0)
Rhinoconjunctivitis	6	(0.2)
Physician-diagnosed asthma	3	(0.1)
Current treatment for asthma	2	(0.1)

* Number of individuals with missing data regarding that variable.

Figure S1: Distribution of the included elders in each region across mainland Portugal



Output 3:

Prevalence of asthma and its association with rhinitis in the elderly

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Prevalence of asthma and its association with rhinitis in the elderly

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KEYWORDS
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Summary
Background: Asthma and rhinitis are frequent respiratory diseases in children and adults. Despite the increase in the aging population, there are few epidemiologic data on both diseases in the elderly. So far, no population-based study has analyzed the association between asthma and rhinitis symptoms and severity in this age group. This study aimed to estimate the prevalence of physician-diagnosed asthma in the population aged ≥65 years in mainland Portugal and to evaluate its association with the presence and classification of rhinitis according to ARIA recommendations, in this age group.
Methods: A cross-sectional, nationwide, population-based survey of individuals aged ≥65 years, living in mainland Portugal was performed.
Results: Data were obtained from 3678 respondents. The prevalence of physician-diagnosed asthma was 10.9% (95% confidence interval (95%CI) 9.9–11.9). The frequency of asthma diagnosis increased with the number of nasal symptoms ($p < 0.001$). A strong association between asthma and rhinitis was found (odds ratio (OR) 13.86 (95%CI 10.66–18.02)). The strength of this association increased with the persistence and severity of rhinitis, being particularly high in elderly subjects with moderate-severe persistent rhinitis (OR 39.9 (95%CI 27.5–58.0)).

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Conclusions: Asthma is common in the elderly and strongly associated with rhinitis. The OR for asthma is especially high in persistent and severe ARIA classification rhinitis types. This study strengthens the need for an integrated assessment of asthma together with rhinitis in the elderly.

Introduction

Asthma and rhinitis are common and they are often associated in children and adults [1—3]. Rhinitis is a risk factor for asthma, being associated with increased severity and health resource use in asthma. Therefore, international guidelines consensually recommend that asthma and rhinitis should be considered together [1,2].

In recent decades, a significant increase in the elderly population has occurred, both in Europe and the U.S. This increase in life expectancy joins a growing concern for healthy, active aging. Epidemiological studies at the population level have been critical to the assessment of population's needs with regard to chronic respiratory diseases [3]. However, elderly-targeted, population-based studies considering asthma and rhinitis are scarce [4—7], although the greatest burden of asthma deaths has occurred among elderly subjects [8,9].

We have recently estimated the prevalence of rhinitis in the elderly population in Portugal and, for the first time, rhinitis has been classified according to Allergic Rhinitis and its Impact on Asthma (ARIA) recommendations in this age group [6]. This study showed that rhinitis was a highly prevalent but underdiagnosed and undertreated disease in the elderly population, despite that over 40% had moderate-severe disease. These data reinforced the need to further explore the association between asthma and rhinitis symptoms and severity in this age group, which, to our knowledge, has not been addressed before in any population-based study targeting the elderly.

Thus, we aimed to estimate the prevalence of physician-diagnosed asthma (PDA) in the population aged ≥65 years in mainland Portugal and to analyze its association with the presence and classification of rhinitis according to ARIA recommendations [2]. The secondary aim was to describe socio-demographic characteristics associated with asthma diagnosis and treatment, in this age group.

Methods

Study design and participants

Population-based, cross-sectional survey applied by face-to-face interview to individuals aged ≥65 years, living in mainland Portugal.

The study's methodology has been previously detailed [6]. Briefly, a stratified (by gender, age and region), multistep sampling strategy was used. The estimation of the number of needed participants was based on the data from the 2001 Portuguese Census on individuals aged ≥65 years, considering all five regions of mainland Portugal [10]. The selection criteria of municipalities within each Portuguese region corresponded to the aging index, population density and purchasing power, indicators that summarize the main regional social disparities [10]. The final selection was done by random-route methodology, where households and nursing homes from the selected municipalities were included. This sampling strategy allowed for a representative sample of the mainland Portuguese population aged ≥65 years, regarding the factors used for sample stratification [6].

Informed consent was obtained from all participants. This study's protocol was reviewed and approved by the Ethics Committee of CUF-Descobertas Hospital, Lisbon, Portugal.

Instruments and data collection

The instrument for data collection has been previously published [6]. The questions on PDA and physician-diagnosed asthma on current treatment (PDA/CT) were based on the European Community Respiratory Health Survey (ECRHS) [11].

Rhinitis severity was assessed using a 0—10 visual analogue scale (VAS), as previously suggested [12].

Questionnaires were applied by trained interviewers between May and July 2008. Inter-interviewer variability in the results was controlled by standardized training and interview framework, which application was monitored by a supervisor.

Definitions

Definitions used in this study are presented in Table 1. In individuals with rhinitis, it was further classified into intermittent/persistent and mild/moderate-severe, according to current ARIA guidelines [2] (Table 1).

Socio-demographic characteristics included gender ("male" or "female") and age (in years); municipality was considered as "urban" or "rural", according to living municipality typology [10], and residency was categorized as living in "own house" (i.e., home ownership), living "with relatives" (i.e., in family members' home ownership) or in "nursing home" (i.e., home for the elderly).

Sample size and statistical analysis

The primary endpoint of the study was rhinitis prevalence [6]. Considering that the prevalence of asthma in the elderly is estimated to be around 8% [13], the inclusion of 3500 subjects (computed based on rhinitis endpoints) allows the estimation of asthma prevalence with an error of 0.9% (precision) in 95% confidence interval (95%CI).

Table 1 Definitions used in this study.

Variable	Source	Definition
Physician-diagnosed asthma (PDA)	Adapted from ECRHS [11]	Positive answer to the question "Has a doctor ever said you have asthma?".
Physician-diagnosed asthma on current treatment (PDA/CT)	Adapted from ECRHS [11]	Presence of physician-diagnosed asthma and a positive answer to the question "Do you take medication for asthma?".
Current rhinitis	Based on ECRHS and ARIA [2,11,43]	Presence, usually or in the last 12 months, of at least two of the following symptoms: "repeated sneezing and itchy nose"; "blocked nose for more than one whole hour" or "runny nose when not having a cold or flu".
Intermittent rhinitis	Built according to ARIA [2]	Current rhinitis with nasal symptoms lasting less than 4 days in a week or lasting more than 4 days/week but less than 4 consecutive weeks.
Persistent rhinitis	Built according to ARIA [2]	Current rhinitis with nasal symptoms lasting for at least 4 days in a week and for more than 4 consecutive weeks.
Mild rhinitis	Adapted from Bousquet et al. [12]	Current rhinitis that had a visual analogue scale severity score ranging between 0 and 5.
Moderate-severe rhinitis	Adapted from Bousquet et al. [12]	Current rhinitis that had a visual analogue scale severity score ranging between 6 and 10.
Rhinoconjunctivitis	Adapted from ECRHS [11]	Presence of rhinitis and a positive answer to the question "Do nasal symptoms usually occur along with red or itchy-watery eyes?".
Physician-diagnosed rhinitis	Additional question [43]	Positive answer to the question "Has a doctor ever said you have rhinitis?".
Current rhinitis treatment	Adapted from ECRHS [11]	Positive answer to the question "Have you had any medication for rhinitis (nasal topical drug or pill) in the last 12 months?".

Categorical variables were described using absolute and relative frequencies with 95%CI; comparisons were performed using the Pearson's chi-square test and linear by linear association. Continuous variables were described with mean and standard deviation (SD); comparisons were performed using an independent samples *t*-test, or Welch test if equality of variances was not assumed. A significance level of 5% was considered.

Univariate logistic regression models for "PDA" and "PDA/CT" were developed using independent variables as risk/predictive factors. Gender, age group, municipality typology and residency were considered as possible risk

Table 2 Socio-demographic characteristics of the participants, including stratification by "physician-diagnosed asthma" status.

	Total (n = 3678) n (%)		Physician-diagnosed asthma			
			Yes (n = 401) n (%)		No (n = 3274) n (%)	
Gender						
Female	2151	(58.5)	254	(63.3)	1896	(57.9)
Age, *years*						
65–74	2128	(57.9)	232	(57.9)	1896	(57.9)
75–84	1204	(32.7)	126	(31.4)	1077	(32.9)
≥85	346	(9.4)	43	(10.7)	301	(9.2)
Municipality						
Urban	2752	(76.5)	288	(71.8)	2461	(75.2)
Rural	845	(23.5)	107	(26.7)	738	(22.5)
Residency						
Own house	2657	(73.2)	254	(63.3)	2400	(73.3)
With relatives	632	(17.4)	81	(20.2)	551	(16.8)
Nursing home	343	(9.4)	64	(16.0)	279	(8.5)

Table 3 Prevalence of physician-diagnosed asthma and physician-diagnosed asthma on current treatment and logistic regression models with crude and adjusted odds ratio (OR) with 95% confidence intervals (95%CI).

	Physician-diagnosed asthma						Physician-diagnosed asthma on current treatment					
	Prevalence		Crude		Adjusted		Prevalence		Crude		Adjusted	
	%	(95%CI)	OR	(95%CI)	OR	(95%CI)	%	(95%CI)	OR	(95%CI)	OR	(95%CI)
Whole sample	10.9	(9.9–11.9)					7.6	(6.7–8.4)				
Gender			0.037*		0.097*				0.046*		0.162*	
Female	11.8	(10.5–13.3)	1.00		1.00		8.3	(7.1–9.5)	1.00		1.00	
Male	9.6	(8.3–11.4)	0.80	(0.64–0.99)	0.83	(0.67–1.03)	6.6	(5.4–7.9)	0.77	(0.60–1.00)	0.83	(0.64–1.08)
Age, years			0.569*		NI				0.531*		NI	
65–74	10.9	(9.8–12.5)	1.00				7.6	(6.5–8.7)	1.00			
75–84	10.5	(8.7–12.3)	0.96	(0.76–1.20)			7.1	(5.7–8.6)	0.93	(0.71–1.23)		
≥85	12.4	(9.0–16.1)	1.17	(0.83–1.65)			9.0	(6.0–12.0)	1.19	(0.80–1.79)		
Municipality			0.076*		0.102*				0.214*		0.299*	
Urban	10.5	(9.4–11.7)	1.00		1.00		7.3	(6.3–8.3)	1.00		1.00	
Rural	12.7	(10.5–15.1)	1.24	(0.98–1.57)	1.22	(0.96–1.55)	8.6	(6.7–10.5)	1.19	(0.90–1.58)	1.16	(0.88–1.54)
Residency			<0.001*		<0.001*				<0.001*		<0.001*	
Own house	9.6	(8.5–10.8)	1.00		1.00		6.2	(5.3–7.1)	1.00		1.00	
With relatives	12.8	(10.2–15.5)	1.39	(1.06–1.81)	1.36	(1.04–1.78)	10.1	(7.6–12.5)	1.71	(1.27–2.32)	1.66	(1.23–2.26)
Nursing home	18.7	(14.2–22.6)	2.17	(1.60–2.93)	2.06	(1.51–2.79)	14.3	(10.6–18.0)	2.53	(1.80–3.56)	2.37	(1.67–3.35)
Current rhinitis			<0.001*		NI				<0.001*		NI	
No	2.9	(2.3–3.6)	1.00				2.1	(1.6–2.7)	1.00			
Yes	29.6	(27.0–32.4)	13.86	(10.66–18.02)			20.5	(18.1–22.9)	12.08	(8.88–16.41)		
Rhinoconjunctivitis			<0.001*		NI				<0.001*		NI	
No	4.3	(3.6–5.1)		1.00			2.7	(2.1–3.3)	1.00			
Yes	36.4	(32.9–39.8)	22.15	(17.53–27.98)			26.6	(23.4–29.8)	16.92	(12.36–23.18)		
Physician-diagnosed rhinitis			<0.001*		NI				<0.001*		NI	
No	7.4	(6.6–8.5)	1.00				5.3	(4.5–6.1)	1.00			
Yes	34.1	(30.1–38.7)	6.46	(5.13–8.13)			23.1	(19.3–26.9)	5.40	(4.15–7.03)		
Current rhinitis treatment			<0.001*		NI				<0.001*		NI	
No	7.6	(6.7–8.5)	1.00				5.5	(4.7–6.3)	1.00			
Yes	32.8	(28.6–37.0)	5.96	(4.74–7.51)	NI		21.5	(17.9–25.1)	4.75	(3.65–6.19)	NI	

* *p*-value; OR, odds ratio; 95%CI, 95% confidence interval; NI, Not included.

factors; and current rhinitis, rhinoconjunctivitis, physician-diagnosed rhinitis, current rhinitis treatment and rhinitis classification categories as possible predictive factors (frequently associated pathologies that may share common risk factors). Multivariate logistic regression models were developed for "PDA" and "PDA/CT". Only possible risk factors were included in the multivariate logistic regression models; they were chosen according to the *p*-value of the univariate analysis, considering a cut-off of <0.250. The Hosmer–Lemeshow test was used to assess calibration of the multivariate models; a $p > 0.05$ was deemed necessary to consider that the model was calibrated. Results of both univariate and multivariate logistic regression models were presented as odds ratio (OR) with 95%CI.

Data analyses were performed using SPSS® version 19.0 for Windows (IBM SPSS, Chicago, IL, USA).

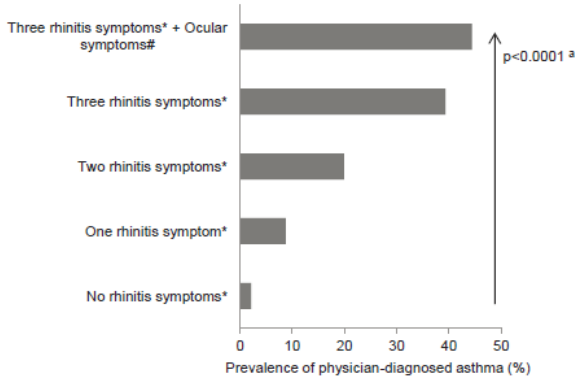
Results

Participants' characteristics

A total of 3699 out of 4000 invited subjects answered the questionnaire (response rate 92.5%). There was no evidence of different response rates according to age. From the answered questionnaires, 21 were excluded: 13 subjects were aged less than 65 years old and eight had insufficient information due to incomplete interviews. Data was obtained from 3678 responders. Participants' socio-demographic characteristics are summarized in Table 2.

Prevalence of physician-diagnosed asthma and physician-diagnosed asthma on current treatment

The prevalence of PDA was 10.9% (95%CI 9.9%–11.9%). Seventy per cent of the elder subjects with asthma reported to be under current asthma treatment. The prevalence of PDA/CT was 7.6% (95%CI 6.7%–8.4%).



(* - Runny nose, Sneezing and itchy nose, Blocked nose; # - Red or itchy-watery eyes; [a] - p-value, linear-by-linear association)

Figure 1 Prevalence of physician-diagnosed asthma in relation to nasal and ocular symptoms.

The prevalence of PDA was lower in men compared to women (9.6% vs 11.8%, $p = 0.037$). No statistically significant differences were found in the prevalence of asthma according to the age group or between urban and rural municipalities (Table 3). Adjusting for other independent variables, living with relatives and, especially, living in a nursing home, when compared with living in their own house, was significantly and positively associated with PDA and PDA/CT (Table 3). In the multivariate analysis, there was no significant effect of gender on both outcomes (Table 3). Table 4 shows the comparison of elderly subjects with PDA according to asthma treatment. Elderly subjects with PDA living in their own house had current asthma treatment less frequently (Table 4).

Table 4 Comparison of elderly subjects with physician-diagnosed asthma, with and without current asthma treatment.

	Physician-diagnosed asthma [$n = 401$]		*p*-Value
	On current treatment [$n = 279$] *n* (%)	No current treatment [$n = 122$] *n* (%)	
Gender			0.608
Female	179 (64.2)	75 (61.5)	
Age, *years*			0.887
65–74	162 (58.1)	70 (57.4)	
75–84	86 (30.8)	40 (32.8)	
≥85	31 (11.1)	12 (9.8)	
Municipality			0.881
Urban	202 (72.4)	86 (70.5)	
Rural	73 (26.2)	34 (27.9)	
Residency			0.020
Own house	164 (58.8)	90 (73.8)	
With relatives	64 (22.9)	17 (13.9)	
Nursing home	49 (17.6)	15 (12.3)	
Current rhinitis	225 (80.6)	100 (82.0)	0.756
Rhinoconjunctivitis	200 (71.7)	72 (59.0)	0.013
Physician-diagnosed rhinitis	111 (39.8)	53 (43.4)	0.493
Current rhinitis treatment	105 (37.6)	53 (43.4)	0.221

Table 5 Prevalence of physician-diagnosed asthma and physician-diagnosed asthma on current treatment according to ARIA rhinitis classification categories.

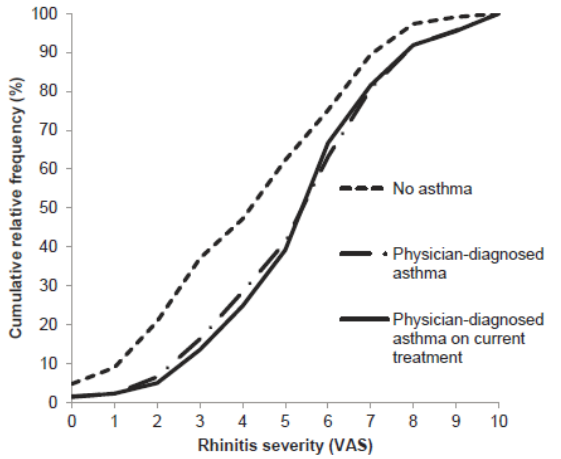
	Physician-diagnosed asthma		Physician-diagnosed asthma on current treatment	
	%	(95%CI)	%	(95%CI)
Mild intermittent rhinitis	<0.0001*		<0.0001*	
No	9.2	(8.2–10.2)	6.6	(5.7–7.5)
Yes	20.2	(16.8–23.6)	13.0	(10.1–15.9)
Moderate-severe intermittent rhinitis	<0.0001*		<0.0001*	
No	9.1	(8.1–10.1)	6.2	(5.4–7.0)
Yes	30.6	(25.4–35.8)	23.2	(18.4–28.0)
Mild persistent rhinitis	<0.0001*		<0.0001*	
No	10.4	(9.4–11.4)	7.2	(6.4–8.0)
Yes	33.3	(22.6–44.0)	24.0	(14.3–33.7)
Moderate-severe persistent rhinitis	<0.0001*		<0.0001*	
No	8.6	(7.7–9.5)	6.0	(5.2–6.8)
Yes	54.8	(47.5–62.1)	37.3	(30.2–44.4)

* *p*-value; 95%CI, 95% confidence interval.

Association between asthma and rhinitis

Of subjects with PDA, 81.1% had current rhinitis, 68.3% rhinoconjunctivitis and 40.9% reported to have physician-diagnosed rhinitis. Current rhinitis was found in 80.7% of patients with PDA/CT, of whom 46.7% had physician-diagnosed rhinitis and 43.6% were on current rhinitis treatment. On the other hand, PDA was reported by 29.6% subjects with current rhinitis, 36.4% with rhinoconjunctivitis and 34.1% with physician-diagnosed rhinitis. In univariate analysis, all three conditions were strongly associated with PDA and PDA/CT, particularly current rhinitis and rhinoconjunctivitis (Table 3). The frequency of rhinoconjunctivitis was higher in subjects with PDA/CT, compared to those without asthma treatment (Table 4).

The prevalence of PDA increased with the number of rhinitis symptoms, from 2.1% in no rhinitis symptoms to 39.3% in three nasal complaints and 44.4% in nasal symptoms combined with ocular symptoms; $p < 0.0001$ by linear-by-linear association (Fig. 1).

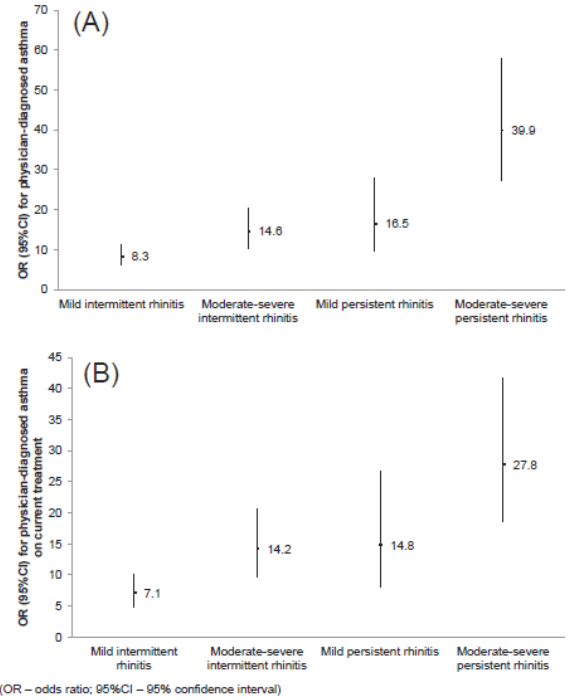


(VAS – visual analogue scale, cm)

Figure 2 Cumulative relative frequency distribution of rhinitis severity visual analogue scale score in elderly subjects, according to the presence of physician-diagnosed asthma and physician-diagnosed asthma on current treatment.

Risk for asthma according to ARIA rhinitis classification

The prevalence of PDA and PDA/CT according to the ARIA classification of rhinitis is presented in Table 5. The



(OR – odds ratio; 95%CI – 95% confidence interval)

Figure 3 Odds ratio with 95% confidence interval for physician-diagnosed asthma (A) and physician-diagnosed asthma on current treatment (B) according to ARIA rhinitis classification categories.

association between PDA and current rhinitis was evident in all rhinitis classes.

Figure 2 shows the cumulative relative frequency distribution of rhinitis severity VAS score, according to the presence of PDA and PDA/CT. Rhinitis severity VAS scores were significantly higher in elderly subjects with PDA (5.7 cm SD 2.1 vs. 4.6 cm SD 2.3 in those without asthma, Welch test $p < 0.001$) and with PDA/CT (5.8 cm SD 2.0 vs. 4.6 cm SD 2.3 in those without asthma, Welch test $p < 0.001$).

The OR for asthma increased with increased frequency and severity of rhinitis symptoms, and was strongest in subjects with moderate-severe persistent rhinitis (Fig. 3A). Having current rhinitis was also significantly associated with PDA/CT; this relation was present in all rhinitis classes, especially in subjects with moderate-severe persistent rhinitis (Table 5; Fig. 3B).

Discussion

A high prevalence of self-reported PDA was found in the Portuguese population aged ≥65 years (10.9%; IC95% 9.9—11.9). This study showed a strong association between rhinitis, rhinoconjunctivitis and asthma diagnosis in this age group. The strength of the association between asthma and rhinitis increased with longer persistence and higher severity of rhinitis symptoms, considering the ARIA rhinitis classification.

This study was the first nationwide population-based epidemiological study exclusively dedicated to the elderly that concurrently evaluated asthma and rhinitis symptoms and ARIA classification and that reported a relation between asthma and rhinitis severity/duration, in this age group. It included a large representative sample of elderly subjects, allowing the estimation of PDA prevalence in mainland Portugal and the characterization of its association with rhinitis according to ARIA recommendations.

The study's main limitations are due to its cross-sectional design, the absence of physician confirmation/evaluation, lung function parameters and *in vivo* and/or *in vitro* atopy markers, and the lack of information regarding environmental exposures (namely tobacco smoke), lower respiratory symptoms, asthma severity and control. Other factors, such as recall or information biases may have influenced the results. Nevertheless, the use of a short and simple questionnaire, applied by trained interviewers, facilitated the inclusion of the elderly subjects. It also minimized difficulties in reading or comprehending and the loss of compliance throughout the interview, allowing for very few missing data regarding all variables [6].

A recent study, the Portuguese National Asthma Survey, estimated that the prevalence of current asthma (i.e., with symptoms in the last 12 months) in Portugal was 6.8% (95%CI 6.0—7.7) [13]. In this study, the subgroup analysis by age groups showed no statistically significant difference in the prevalence of current asthma in individuals aged ≥65 years. However, adjusting for possible confounding factors, the prevalence of asthma was lower in elderly subjects who reported no heart disease. Since heart disease is common in the elderly and may be a cause of respiratory symptoms, the authors pointed out the possibility of an overestimation of current asthma in elderly subjects with cardiac disease. Asthma prevalence estimates did not change after adjusting for chronic bronchitis [13].

In the present study, in spite of the limitations due to PDA being only self-reported and the lack of information on environmental exposures including tobacco smoke, the fact that a doctor's diagnosis and current asthma treatment were considered may have helped to reduce bias and confounding factors related to respiratory symptoms caused by other conditions, such as heart disease, bronchitis or chronic obstructive pulmonary disease (COPD). Nonetheless, distinguishing asthma from COPD can be particularly difficult. COPD is often assumed to be an "aging-related disease", remaining an important comorbidity leading physicians to diagnose COPD rather than asthma in the elderly [14—16]. Conversely, other studies report asthma overdiagnosis in adults, including COPD misdiagnosis with asthma [17—19].

Considering the prevalence of PDA/CT as a proxy for "active asthma", we found that 7.6% (95%CI 6.7—8.4%) of the Portuguese elderly population had an active disease. Despite the lack of information on asthma control, this does not differ significantly from the estimated prevalence of current asthma in elderly in the study mentioned above (8.0% (95%CI 6.7—9.5)) [13], suggesting that this might be an accurate approximation of the real prevalence in the Portuguese population. In other epidemiological studies targeting elderly subjects from other countries, the prevalence of asthma varied between 3.6% and 7.6% [4,5,7,20—27]. However, the different selection criteria and definitions of asthma limit inter-studies comparisons. Asthma overdiagnosis or other stated biases may have concurred for the higher prevalence obtained in this study. A study including medical evaluation, information on tobacco and other environmental exposures, and lung function parameters would be of utmost importance to address this issue in the elderly.

In this study we noted that, as for rhinitis [6], after adjusting for independent variables, the diagnosis of asthma was associated with living with relatives and especially living in a nursing home. This may suggest that these elderly subjects, compared to those who reside in their own house, have greater frequency of asthma or better access to medical care. The fact that those individuals living in their own house had current asthma treatment less frequently may either reflect better asthma control in this group, or, on the contrary, support the assumption regarding worse access to medical care in elderly subjects living in their own house. Yet, it should also be considered that those elderly living in nursing homes often have greater deconditioning or multiple co-morbidities that may mimic asthma, which could contribute for misdiagnosis of this disease.

In accordance with the Portuguese National Asthma Survey [13], in multivariate analysis, asthma prevalence was similar in both genders and according to municipality typology. Unlike suggested by other studies [15,20], the prevalence of asthma in the elderly did not diminish with age. However, this could also reflect an increasing tendency for asthma misclassification with COPD or other mimicking conditions, with increasing age [17—19], which should be explored.

The present study, including only older adults, also showed a strong association between rhinitis, rhinoconjunctivitis and self-reported asthma diagnosis. About 80% of elderly subjects diagnosed with asthma had rhinitis; among elderly subjects with rhinitis, 30% had asthma diagnosed by a doctor. The prevalence of asthma diagnosis increased with the number of nasal symptoms, especially when they were associated with ocular symptoms. These results support an extensive nose—lung interaction also in the elderly, which has been observed by others [28—31]. Furthermore, considering the difficulties in distinguishing asthma from other relevant conditions in differential diagnosis, our data may more broadly indicate that there might also be a relationship between rhinitis and other diseases responsible for lower respiratory symptoms in the elderly, namely COPD. Other studies strengthen the fact that the "united airways" concept goes beyond the scope of asthma and also support an association between rhinitis and COPD [32—34].

The definition for current rhinitis used in this study, requiring the presence of at least two nasal symptoms, suggests the diagnosis of allergic rhinitis. This is reinforced in the elderly with concomitant complaints of red eye, eye pruritus or epiphora, suggesting allergic rhinoconjunctivitis. The fact that the association between rhinitis and asthma has been particularly notable in elderly patients with rhinoconjunctivitis may suggest an increased risk of asthma when there is greater likelihood of involvement of allergic mechanisms. Rhinoconjunctivitis was more frequent in elderly subjects with PDA/CT, compared to those without asthma treatment, which may suggest a possible relation with atopy. However, the definitions of allergic rhinitis and allergic rhinoconjunctivitis were not used because they require the assessment of the specific immunological mechanisms involved and that was not part of this study.

This report further emphasizes that rhinitis in the elderly is underdiagnosed and undertreated [6]. About 40% of elderly patients with PDA had complaints of current rhinitis but were not diagnosed or treated for rhinitis. This is reinforced in those on current treatment for asthma and concurrent current rhinitis, where more than half of these patients had no physician diagnosis of rhinitis. Considering that rhinitis symptoms and lack of control interfere with asthma [1,2], there should be a greater awareness for the assessment of nasal disease in patients with an asthma diagnosis.

The strong association found between asthma, asthma on current treatment and all ARIA classes of rhinitis further supports the importance of an accurate rhinitis diagnosis in the elderly, including in milder presentations. The ARIA classification of rhinitis provided important information with regard to the strength of the association with asthma, as it increased with increased persistency and severity of rhinitis. While contradicting some previous studies [35—37], moderate-severe persistent rhinitis had an especially strong association with asthma, as also reported by other authors, in different age groups [2,38—42].

Addressing respiratory diseases in the elderly, including asthma and rhinitis, is a recognized imperative need [1—3], especially when taking into account their impact and the trend for an increase in the elderly population in many countries worldwide. This study showed that asthma is prevalent in the elderly, being strongly associated with rhinitis and rhinoconjunctivitis. The strength of this association increases very significantly with longer persistence and higher severity of rhinitis. This study highlights the need for a clinical integrated, global assessment of asthma together with rhinitis in this age group and may contribute to the designing of future studies addressing both diseases in the elderly.

Ethics statement

This study was conducted according to the principles of the Helsinki Declaration. Informed consent was obtained from all participants. This study's protocol was approved by the Ethical Review Board of the CUF-Descobertas Hospital, Lisbon, Portugal.

Author contributions

HP participated in data analysis and interpretation and wrote the manuscript draft, AMP participated in data interpretation and review, CN participated in the study conception, JB provided critical review during the project, JAF participated in data analysis and interpretation and provided critical review during the project, MMA coordinated the study participating in all stages and tasks. All authors have reviewed and approved the final manuscript.

Conflict of interest

Mário Morais-Almeida (MMA) reports lecture fees from Astrazeneca, GSK, OM Pharma, Sanofi and Siemens Diagnostics; lecture fees and honoraria for advisory board from FAES Farma; lecture fees and non-financial research project support from MSD; travel grant, lecture fees and honoraria for advisory board from Novartis; and honoraria for advisory board from Pfizer. Jean Bousquet (JB) received honoraria for scientific and advisory boards, lectures during meetings, press conferences from Stallergènes, Actelion, Almirall, AstraZeneca, Chiesi, GSK, Merck, MSD, Novartis, OM Pharma, Sanofi-Aventis, Schering Plough, Teva, Uriach. João Almeida Fonseca (JAF) reports lecture fees and non-financial research project support from MSD, lecture fees and honoraria for advisory board from Novartis and lecture and training fees from GSK. Helena Pité (HP), Ana Margarida Pereira (AMP), and Carlos Nunes (CN) have no conflicts of interest to declare.

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Part 2: Definition of early childhood wheezing phenotypes related to asthma persistence

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Preschool-age wheezing phenotypes and asthma persistence in adolescents

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ABSTRACT

Background: *Predicting long-term outcomes in individuals with childhood wheezing is of major clinical relevance.*

Objective: *To identify and characterize childhood wheezing phenotypes related to asthma persistence in adolescence with a multidimensional statistical model, independent of predefined hypotheses.*

Methods: *This prospective cohort study included 308 children, ages < 7 years, with recurrent wheezing. We systematically evaluated asthma prevalence in children at 3, 8, and 13 years of follow-ups. Risk factors associated with asthma persistence in adolescence were analyzed with multivariable logistic regression. Early childhood wheezing phenotypes were identified with k-means cluster analysis of variables selected with the logistic regression analysis, which were based on questionnaires and skin-prick tests. These phenotypes were compared for predicting asthma prevalence, use of control treatments, and lung function in childhood and adolescence.*

Results: *Asthma prevalence was 58.3% (n = 249) and 53.5% (n = 170) at the 8- and 13-year follow-ups, respectively. Preschool-age diagnoses of atopy (odds ratio 11.8 [95% confidence interval, 4.0–34.6]) and rhinitis (odds ratio 10.4 [95% confidence interval, 3.7–29.1]) were independent risk factors for asthma persistence in adolescence. We identified three early childhood wheezing phenotypes: transient, persistent atopic, and persistent nonatopic. The latter two were characterized by rhinitis during preschool age. These phenotypes could predict the following outcomes: asthma symptom persistence, use of control treatments, and lung function during childhood and adolescence (p < 0.03).*

Conclusion: *Asthma persistence through adolescence reflected different wheezing phenotypes based on preschool-age comorbidities, particularly rhinitis, with or without atopy. Our results supported that wheezing phenotypes, identified at early ages from simple measurements, could predict asthma and lung function outcomes.*

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Wheezing in early childhood is among the most frequent respiratory symptoms.[1,2] Many children with early wheezing become symptom free in later childhood and adolescence. However, early onset wheezing is also associated with persistent asthma in adulthood and with more severe, persistent lung function impairments.[3–5] Wheezing phenotype definitions that can predict long-term outcomes are of major clinical relevance because they provide a basis for informed counseling to parents and for improved targeted management.[6,7] Currently, to our knowledge, no consensual classification exists.[8]

The main aims of the present study were to identify and characterize early childhood wheezing phenotypes in a multidimensional statistical model, independent of predefined hypothesis, based on observable data. We developed a model that combined different patient characteristics to assess the prognosis of asthma persistence for different groups. This model considered the symptoms, the use of control treatments, and lung function parameters in school-age and adolescent children.

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METHODS

Study Design and Participants

During a 12-month period in 1993, a cohort of 308 children was selected from consecutive patients during their first visits to a children's hospital immunoallergy outpatient clinic in Lisbon, Portugal.[9] Inclusion criteria were the following: age < 7 years old and a medical diagnosis of recurrent wheezing (Fig. 1).[9] Children with diagnosed congenital or perinatal diseases, including cardiopulmonary diseases, pneumonia or pneumonitis, aspiration, cystic fibrosis, immunodeficiency, or gastroesophageal reflux disease were excluded.

Systematic follow-up evaluations were performed after 3, 8, and 13 years. The follow-up algorithm is presented in Fig. 1. During follow-up, children could be treated according to their physician's decision; there was no therapeutic interventional schedule in

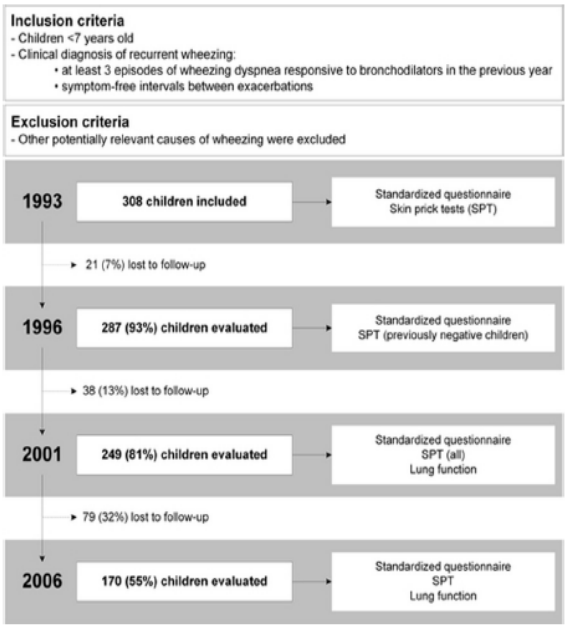

Figure 1. Follow-up study algorithm.

this study. Informed consent was obtained from each child's parents, and the study was approved by CUF Descobertas Hospital Ethics Committee. Authors' contributions included the following: H. Pité participated in data analysis and interpretation and wrote the manuscript draft. A. Gaspar participated in study conception, data analysis, and provided critical review during the project. M. Morais-Almeida coordinated the study and participated in all stages and tasks. All authors reviewed and approved the final manuscript.

Instruments and Data Collection

Questionnaire. The instrument for data collection was adapted from the "International Study of Asthma and Allergies in Childhood" questionnaire.[10] Additional questions were included, regarding age of wheezing onset, food allergies, parental asthma, exposure to tobacco smoke, family pet ownership, and day care attendance. The questionnaires were administered by trained medical physicians at each visit.

Skin-Prick Tests. Skin-prick tests were performed according to international recommendations.[11,12] The skin was pricked with steel lancets (Prick Lancetter, Hollister-Stier Laboratories, Spokane, WA), and the following allergen extracts were applied: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Parietaria*, grass pollen mixture, tree pollen mixture, fungi mixture, dog and cat epithelium (Allergopharma Joachim Ganzer KG, Reinbek, Germany); *Blatella germanica*, *Periplaneta americana*, and *Blatta orientalis* (CBF Leti, Madrid, Spain); and cow's milk, egg, fish, and wheat (Stallergènes Group, Antony, France). Histamine hydrochloride (10 mg/mL) was used as a positive control; a saline solution was the negative control. A wheal mean diameter of >3 mm was considered a positive test.

Lung Function Tests. Lung function was evaluated at the 8- and 13-year follow-ups. Spirometry and a bronchodilation test (pneumotachograph Vitalograph Compact II; Vitalograph Ltd, Buckingham, England) were applied according to international recommendations.[13,14] These tests were performed outside exacerbation episodes.

Definitions

We defined recurrent wheezing in Fig. 1. Children were considered asymptomatic when they had no symptoms in the previous 12 months, without the use of any asthma control therapy. A clinical diagnosis of asthma was made according to medical criteria of a characteristic history and examination of respiratory symptoms that vary over time and intensity, based on existing guidelines,[15] after exclusion of other possible causes of respiratory symptoms. The asthma diagnosis was considered from the 3-year follow-up and confirmed with variable airflow limitation after the age of 6 years.

We determined the presence of rhinitis, eczema, food allergy, and recurrent respiratory infections based on questionnaire answers, clinical files, and medical examination: rhinitis was defined as the presence of nasal itching, rhinorrhea, sneezing and/or nasal obstruction that occurred persistently during a minimum period of 4 weeks, excluding infectious episodes; eczema was defined as clinical evidence of atopic dermatitis, according to age group criteria[9]; food allergy was defined as a reproducible set of signs and symptoms of immune hypersensitivity reaction after food ingestion, and recurrent respiratory infections defined as more than six respiratory infection episodes per year. These conditions were validated by a medical specialist. Parental asthma, tobacco exposure, and use of control treatments were self-reported. Atopy was diagnosed when at least one skin-prick test was positive. Bronchial obstruction and a positive bronchodilation test were defined according to international standards.[13,14]

Statistical Analysis

Continuous variables are expressed as the mean and standard deviation. Categorical variables are expressed as absolute and relative frequencies. Comparisons were performed with the Pearson χ^2 test and with generalized estimating equations for longitudinal

Table 1 **Participant characteristics at each evaluation time point**

	Enrollment (n = 308)	3 Years of Follow-up (n = 287)	8 Years of Follow-up (n = 249)	13 Years of Follow-up (n = 170)	p Value
Age, mean (SD), y	3.7 ± 1.7	7.0 ± 1.8	11.1 ± 1.8	16.0 ± 1.8	—
Age at enrollment <36 mo, no. (%)	108 (35.1)	103 (35.9)	89 (35.7)	71 (41.8)	***0.019***
Boys, no. (%)	187 (60.7)	177 (61.7)	155 (62.2)	107 (62.9)	0.227
Age of wheezing onset of <36 mo, no. (%)	229 (78.2)	218 (79.0)	194 (78.5)	134 (79.3)	0.351
Paternal asthma, no. (%)	41 (13.5)	36 (12.7)	31 (12.4)	23 (13.5)	0.514
Maternal asthma, no. (%)	55 (18.2)	53 (18.7)	47 (18.9)	30 (17.6)	0.818
Personal history at preschool age, no. (%)					
Rhinitis	184 (59.7)	176 (61.3)	156 (62.7)	103 (60.6)	0.071
Eczema	67 (21.8)	62 (21.6)	61 (24.5)	41 (24.1)	0.055
Food allergy	17 (5.5)	16 (5.6)	16 (6.4)	15 (8.8)	***0.001***
Atopy	148 (48.1)	142 (49.5)	121 (48.6)	79 (46.5)	0.999
Family pet ownership in the first 12 mo of life, no. (%)	66 (26.5)	65 (27.2)	66 (26.5)	49 (29.2)	0.153
Day care attendance before 12 mo of age, no. (%)	71 (28.5)	66 (27.6)	71 (28.5)	54 (32.1)	0.054
Maternal smoking during pregnancy, no. (%)	35 (14.1)	33 (13.8)	35 (14.1)	21 (12.5)	0.327

SD = standard deviation.
Bold values denote statistically significant differences.

analyses of categorical data; $p < 0.05$ was considered significant.

Logistic regression models for persistent asthma in adolescence were developed with independent variables as possible risk factors. In the univariable analysis, all study variables that might be related to asthma persistence were comprehensively included, regardless of the relationship between them and the evaluation time point. In the multivariable logistic regression model, variables related to the first evaluation time point were preferably selected (*i.e.*, we excluded variables evaluated only at the third and fourth time points). Variables were included that had achieved $p < 0.250$ in the univariable analysis. Results of both univariable and multivariable logistic regression models are presented as the odds ratio with 95% confidence interval. To identify phenotypes, the k-means method was used as the primary clustering technique. This method was applied to a set of variables selected based on logistic regression analysis for asthma persistence in adolescence. Data analyses were performed with SPSS version 19.0 for Windows (IBM SPSS, Chicago, IL).

RESULTS

Participant Characteristics

The number of children observed at each follow-up time point is indicated in Fig. 1. Most baseline characteristics were similar among participants at the different follow-up time points. However, in the population group evaluated at the last follow-up time point, we observed slightly higher frequencies of children ages of <36 months at enrollment and children who had developed a food allergy by preschool age (Table 1).

Asthma Prevalence at School Age and Adolescence

The asthma prevalence at each evaluation time point, including children who completed 13 years of follow-up is shown in Table 2. Asthma prevalence was significantly higher in children who were ages ≥ 3 years at enrollment; had a maternal asthma history; had a personal history of rhinitis, eczema, and atopy at preschool ages; used a control treatment; showed bronchial obstruction; and had a positive bronchodilation test (Table 2). Skin-prick tests at preschool age were positive for house-dust mites (46.4%), pollens (3.9%), animals (2.6%), and fungi (1.0%). Sensitization to food occurred in 3.9% of children. Control treatment after 8 and 13 years of follow-up consisted of inhaled corticosteroids, 14.9% and 44.1%, respectively; inhaled corticosteroid associated with long-acting β2-agonist, 18.5% and 24.1%, respectively; montelukast, 8.0% and 14.1%,

Table 2 Asthma prevalence at each evaluation time point (total and stratified by groups), when considering participants after 13 years of follow-up (n = 170)

	3 Years of Follow-up (%)	8 Years of Follow-up (%)	13 Years of Follow-up (%)	p Value
Total no.	66.1	58.3	53.5	0.001
Age at enrollment	0.100*	0.064*	0.029*	
<36 mo	58.8	50.0	43.7	0.009
≥36 mo	71.1	64.3	60.6	0.021
Sex	0.828*	0.304*	0.930*	
Girls	65.0	53.2	54.0	0.072
Boys	66.7	61.3	53.3	0.003
Age of wheezing onset	0.023*	0.003*	0.097*	
<36 mo	61.5	52.3	50.0	0.006
≥36 mo	82.4	80.0	65.7	0.026
Paternal asthma	0.123*	0.053*	0.448*	
No	63.9	55.5	52.4	0.003
Yes	81.0	77.3	60.9	0.014
Maternal asthma	0.614*	0.002*	0.046*	
No	65.2	52.9	50.0	<0.001
Yes	70.0	83.3	70.0	0.999
Exposure to ETS at home (3 y of follow-up)	0.624*	0.334*	0.416*	
No	67.9	54.4	56.8	0.027
Yes	64.3	61.8	50.6	0.008
Exposure to ETS at home (8 y of follow-up)	0.393*	0.272*	0.472*	
No	68.5	54.4	55.6	0.007
Yes	62.2	62.8	50.0	0.030
Maternal smoking during pregnancy	0.786*	0.906*	0.953*	
No	65.3	58.5	53.1	0.002
Yes	68.4	57.1	52.4	0.102
Family pet ownership in the first 12 mo of child's life	0.255*	0.886*	0.314*	
No	68.4	58.0	55.5	0.004
Yes	59.2	59.2	46.9	0.05
Day care attendance	0.004*	0.004*	0.127*	
No attendance or only after 12 mo	73.0	65.8	57.0	0.001
Attendance before 12 mo	50.0	42.6	44.4	0.255
Personal history of rhinitis at preschool age	<0.001*	<0.001*	<0.001*	
No	31.8	20.9	13.4	0.004
Yes	88.9	83.2	79.6	0.032
Personal history of eczema at preschool age	0.001*	<0.001*	<0.001*	
No	59.5	48.0	45.7	0.003
Yes	87.2	90.2	78.0	0.035
Personal history of food allergy at preschool age	0.657*	0.299*	0.599*	
No	65.6	57.1	52.9	0.001
Yes	71.4	71.4	60.0	0.335
Controller treatment (8 y of follow-up)#	<0.001*	<0.001*	<0.001*	
No	39.5	19.2	6.4	<0.001
Yes	88.4	92.1	93.3	0.208
Controller treatment (13 y follow-up)#	<0.001*	<0.001*	<0.001*	
No	43.0	28.1	11.2	<0.001
Yes	91.0	92.3	100.0	0.001
Atopy at preschool age	<0.001*	<0.001*	<0.001*	
No	39.8	40.0	25.3	0.014
Yes	96.1	79.5	86.1	0.017

Continued

Table 2 Continued

	3 Years of Follow-up	8 Years of Follow-up	13 Years of Follow-up	*p* Value
Bronchial obstruction (8 y of follow-up)	0.067*	**<0.001***	**0.006***	
No	60.9	46.4	45.5	**0.001**
Yes	75.5	82.1	67.9	0.172
Bronchial obstruction (13 y of follow-up)	0.504*	0.055*	**0.034***	
No	65.6	57.1	52.1	**<0.001**
Yes	80.0	100.0	100.0	**<0.001**
Bronchodilation test (8 y of follow-up)	**0.014***	**<0.001***	**<0.001***	
Negative	59.6	47.5	43.2	**<0.001**
Positive	79.6	84.0	76.0	0.396
Bronchodilation test (13 y of follow-up)	0.859*	0.225*	0.181*	
Negative	86.8	85.7	83.1	0.481
Positive	88.9	100.0	100.0	**0.010**

ETS = environmental tobacco smoke.
**The p values for the difference between classes, when considering classes in each evaluation time point.*
#When considering treatment with inhaled corticosteroids, leukotriene receptor antagonist, or specific immunotherapy.
Bold values denote statistically significant differences.

respectively; and/or allergen-specific immunotherapy, 6.8% and 6.5%, respectively.

Risk Factors for Asthma Persistence in Adolescence

Asthma persistence in adolescence was associated with age ≥ 3 years at the time of enrollment; personal history of rhinitis, eczema, and atopy at preschool ages; control treatment use; and abnormal lung function parameters at school age (Table 3). A personal history of rhinitis and atopy at preschool ages were confirmed as independent risk factors for asthma persistence in adolescence (Table 3).

Phenotype Identification

The sample used to define phenotypes consisted of 247 children with recurrent wheezing at preschool ages (when considering the participants who were not missing values in all variables used to define the clusters); 153 (61.9%) were boys, and the mean (standard deviation) age was 3.6 ± 1.6 years at enrollment. Variables used to define the phenotypes included age of wheezing onset; maternal asthma; paternal asthma; day care attendance; and personal histories of rhinitis, eczema, and atopy at preschool ages. The main characteristics of these phenotypes are summarized in Table 4. The three phenotypes were called atopic persistent wheezing, nonatopic persistent wheezing, and nonatopic transient wheezing based on the most pertinent characteristics.

Cluster Phenotype 1: Atopic Persistent Wheezing

This group was essentially characterized by the presence of both rhinitis and atopy at preschool age. The frequency of this phenotype was 44% at enrollment.

Cluster Phenotype 2: Nonatopic Persistent Wheezing

These children were characterized by the presence of rhinitis, despite a lack of atopy, and an early wheezing onset. Most children in this group had a personal history of eczema and maternal asthma. The frequency of this phenotype was 14% at enrollment.

Cluster Phenotype 3: Nonatopic Transient Wheezing

Despite an early wheezing onset and the absence of atopy, most of these children had no history of rhinitis, eczema, or food allergy at preschool ages and no maternal history of asthma. The frequency of this phenotype was 42% at enrollment.

Comparing Prognoses Across Identified Phenotypes

The frequency of asthma persistence was consistently higher among children with persistent wheezing compared with those with transient wheezing, across all evaluation time points. Similarly, the use of control treatments was significantly different among the groups (Table 5). The cumulative asthma frequency according to age for the different wheezing phenotypes is shown in Fig. 2. Children with nonatopic persistent wheezing had a higher frequency of bronchial obstruction at school age and adolescence compared with children with the transient phenotype. A positive bronchodilation test was more common in children with atopic persistent wheezing at school age and in children with nonatopic persistent wheezing in adolescence (Table 5).

Table 3 **Logistic regression models for asthma persistence in adolescence; unadjusted and adjusted ORs with 95% CIs are presented**

	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Age at enrollment of ≥36 mo	**1.99 (1.07–3.68)**	0.78 (0.26–2.27)
Boys (vs girls)	0.97 (0.52–1.82)	NI
Age of wheezing onset of ≥36 mo	1.92 (0.88–4.16)	0.91 (0.27–3.09)
Family history of allergic disease		
Maternal asthma	2.33 (0.99–5.45)	2.64 (0.76–9.22)
Paternal asthma	1.41 (0.58–3.47)	1.12 (0.29–4.23)
Exposure to ETS smoke at home: yes, ≥1 smoker (vs not exposed)		
With 3 y of follow-up	0.78 (0.43–1.43)	NI
With 8 y follow-up	0.80 (0.44–1.47)	NI
Maternal smoke during pregnancy, yes	0.95 (0.38–2.37)	NI
Animal contact		
Yes (any)	0.93 (0.50–1.75)	NI
At home	0.92 (0.50–1.70)	NI
In the first 12 mo of life	0.71 (0.36–1.39)	NI
Day care attendance		
Yes (any time)	0.74 (0.36–1.51)	NI
Yes, before 12 mo of age (vs no attendance or after 12 mo of age)	0.60 (0.31–1.16)	0.85 (0.29–2.56)
History of allergic disorder (at enrollment)		
Rhinitis (vs no rhinitis)	**25.16 (10.75–58.89)**	**10.43 (3.74–29.05)**
Eczema (vs no eczema)	**4.22 (1.86–9.55)**	2.35 (0.81–6.83)
Food allergy (vs no food allergy)	1.34 (0.45–3.93)	NI
Recurrent respiratory infections, yes	0.98 (0.45–2.12)	NI
Asthma controller treatment (8 y of follow-up)*		
Yes (any)	**201.97 (59.17–689.44)**	NI
Specific immunotherapy	**12.16 (1.54–95.77)**	NI
Atopy (at enrollment)	**18.28 (8.27–40.40)**	**11.81 (4.03–34.64)**
Lung function		
Bronchial obstruction (8 y of follow-up)	**2.53 (1.29–4.95)**	NI
Positive bronchodilation test (8 y of follow-up)	**4.16 (1.98–8.76)**	NI

OR = odds ratio; CI = confidence interval; NI = not included; ETS = environmental tobacco smoke.
**When considering treatment with inhaled corticosteroids, leukotriene receptor antagonist, or specific immunotherapy.*
Bold values denote statistically significant differences.
Hosmer and Lemeshow 0.693; area under the curve 0.914.

DISCUSSION

This cohort study revealed three distinct early childhood wheezing phenotypes based on a multidimensional, hypothesis-free statistical model. These phenotypes were predictive of asthma persistence through school age and adolescence. These defined phenotypes included combined features from other previously described phenotypic classifications. Both atopy and rhinitis during preschool ages were independent risk factors for asthma persistence.

To date, this study represented the largest cohort study in Portugal conducted on children with recurrent wheezing. Our application of hypothesis-free statistical models to this cohort was important in validating previous phenotypic classifications. This study also had the advantage of a long follow-up time. We analyzed data for a large number of patient characteristics, including respiratory symptoms, personal and family histories of allergic diseases, environmental exposures, skin-prick testing, and lung function evaluations. All data regarding diagnoses were validated by allergy specialists. The children were systematically evaluated at key moments of childhood development up to adolescence. Although the sample selection was based on the first visit to a specialized center, which may be associated with a bias, it provided sample homogeneity with respect to clinical manifestations and wheezing frequency and severity.

Table 4 **Baseline characteristics (at enrollment) of the defined clusters ($N = 247$)**

Characteristics	Cluster 1, no. (%) ($n = 109$)*	Cluster 2, no. (%) ($n = 35$)#	Cluster 3, no. (%) ($n = 103$)§	p Value
Age at enrollment of <36 mo	23 (21.1)	18 (51.4)	47 (45.6)	**<0.001**
Boys	67 (61.5)	25 (71.4)	61 (59.2)	0.434
Age of wheezing onset of <36 mo¶	69 (63.3)	31 (88.6)	94 (91.3)	**<0.001**
Paternal asthma¶	18 (16.5)	3 (8.6)	10 (9.7)	0.244
Maternal asthma¶	12 (11.0)	26 (74.3)	8 (7.8)	**<0.001**
Maternal smoke during pregnancy	19 (17.4)	4 (11.4)	12 (11.7)	0.426
Family pet ownership in the first 12 mo of patient's life	24 (22.0)	13 (37.1)	29 (28.2)	0.194
Day care attendance before 12 mo¶	28 (25.7)	4 (11.4)	38 (36.9)	**0.011**
Personal history at preschool age				
Rhinitis¶	105 (94.5)	32 (91.4)	18 (17.5)	**<0.001**
Eczema¶	30 (27.5)	26 (74.3)	5 (4.9)	**<0.001**
Food allergy	6 (5.5)	7 (20.0)	3 (2.9)	**0.002**
Atopy¶	105 (96.3)	8 (22.9)	7 (6.8)	**<0.001**

**Atopic persistent wheezing.*
#Nonatopic persistent wheezing.
§Nonatopic transient wheezing.
¶Variables that were used to define the clusters.
Bold values denote statistically significant differences.

The main limitations of this study were the loss of participants during follow-up and the lack of objective markers for environmental exposures (*viz.* tobacco smoke) and control treatment use. At the end of 13 years of follow-up, 55% of the cohort was lost to follow-up. However, the cohort remained homogenous in the main baseline characteristics throughout all evaluation time points. Although atopy at preschool age was defined by skin-prick test results to a broad number of possible clinically relevant set of aeroallergens and food allergens, these were not fully comprehensive and no other skin or blood test results were considered. In some cases, allergen mixtures were used to limit the number of skin-prick tests to perform in young children. The use of additional tests and allergens, *viz.* individual molds, could have brought additional information,[16] and possibly influence results regarding atopy classification.

Longitudinal birth-cohort studies have made the largest contributions to identifying early childhood wheezing phenotypes.[2–4,17–19] However, many phenotypic classifications have been limited to single disease dimensions, which were subjectively defined, based on directly observable characteristics. This approach has generated distinct classification systems, which were not strictly comparable. Moreover, these classifications were not generalizable to different age groups, and the incorporation of other characteristics was constrained.[20,21] Temporal criteria limit the clinical use of phenotypic classifications that can only be established retrospectively.[20,21] The European Respiratory Society Task Force proposed the classification of episodic viral wheeze and multiple-trigger wheeze.[22] This classification has been revised due to difficulties in including children in mutually exclusive groups and to the rapid symptom pattern change over time.[8] The international consensus group review acknowledges that this temporal classification of wheeze is a relatively poor predictor of long-term outcome.[8]

To overcome these difficulties, it has been more recently proposed that statistical methods that can simultaneously account for multiple disease dimensions may facilitate the identification of relevant phenotypes.[23] In the present study, children with recurrent wheeze were included, irrespective of any classifications. Phenotypes were treated as an unknown rather than defining them *a priori*. To reduce subjectivity, the set of variables included in the cluster analysis was exclusively derived from the logistic regression model for asthma persistence, which included all study variables. The three defined phenotypes significantly differed in long-term outcomes of wheezing, which is important information for research and daily clinical practice. Consistent with previously described phenotypes,[1,17] we named them "atopic persistent," "nonatopic persistent," and "nonatopic transient" wheezing.

Most children remained symptomatic throughout the study, in contrast with other reports that stated that 60% of preschool children with wheezing became asymptomatic by age 6 years.[24] This difference was

Table 5 Characteristics and prognosis of the defined clusters when considering individuals evaluated after 13 years of follow-up ($N = 167$)

	Cluster 1, no. (%) ($n = 71$)*	Cluster 2, no. (%) ($n = 24$)#	Cluster 3, no. (%) ($n = 72$)§	p Value
Characteristics				
Age at enrollment, 36 mo	15 (21.1)	13 (54.2)	41 (56.9)	**<0.001**
Boys	44 (62.0)	17 (70.8)	44 (61.1)	0.680
Age of wheezing onset <36 mo¶	44 (62.0)	22 (91.7)	66 (91.7)	**<0.001**
Paternal asthma¶	12 (16.9)	2 (8.3)	8 (11.1)	0.444
Maternal asthma¶	8 (11.3)	16 (66.7)	5 (6.9)	**<0.001**
Exposure to ETS at home (3 y of follow-up)	37 (52.1)	10 (41.7)	41 (56.9)	0.427
Exposure to ETS at home (8 y of follow-up)	32 (45.1)	10 (41.7)	35 (48.6)	0.818
Maternal smoke during pregnancy	11 (15.5)	3 (12.5)	7 (9.7)	0.582
Family pet ownership in the first 12 mo of life	14 (19.7)	11 (45.8)	24 (33.3)	**0.032**
Day care attendance before 12 mo of age¶	22 (31.0)	3 (12.5)	29 (40.3)	**0.040**
Personal history of at preschool age				
Rhinitis¶	67 (94.4)	22 (91.7)	12 (16.7)	**<0.001**
Eczema¶	20 (28.2)	19 (79.2)	2 (2.8)	**<0.001**
Food allergy	5 (7.0)	6 (25.0)	3 (4.2)	**0.005**
Atopy¶	69 (97.2)	5 (20.8)	4 (5.6)	**<0.001**
Prognosis				
Asthma persistence				
3 y of follow-up	67 (97.1)	16 (69.6)	23 (32.9)	**<0.001**
8 y of follow-up	58 (81.7)	22 (91.7)	17 (23.6)	**<0.001**
13 y of follow-up	62 (87.3)	16 (66.7)	10 (13.9)	**<0.001**
Controller treatment				
8 y of follow-up	58 (81.7)	17 (73.9)	13 (18.1)	**<0.001**
13 y of follow-up	52 (74.3)	16 (66.7)	9 (12.5)	**<0.001**
Bronchial obstruction				
8 y of follow-up	27 (38.0)	12 (50.0)	16 (22.2)	**0.021**
13 y of follow-up	1 (1.4)	3 (12.5)	0 (0.0)	**0.002**
Bronchodilation test, positive				
8 y of follow-up	30 (42.5)	8 (33.3)	11 (15.3)	**0.002**
13 y of follow-up	2 (4.3)	5 (33.3)	1 (6.3)	**0.005**

ETS = environmental tobacco smoke.
**Atopic persistent wheezing.*
#Nonatopic persistent wheezing.
§Nonatopic transient wheezing.
¶Variables that were used to define clusters.
Bold values denote statistically significant differences.

most likely due to the distinct sample selection criteria. In the present study, we selected children with at least three wheezing episodes, but the Tucson study only required one episode.[24] However, we could not exclude the possibility that full symptom resolution may have occurred more frequently among the children lost in follow-up. We identified atopy at preschool ages as an independent risk factor for asthma persistence in adolescence. This finding supported previous reports that showed that recurrent wheezing in early childhood, associated with allergen sensitization, predicted a poor prognosis.[6,24–28] It further reinforced the need to perform allergy tests at early ages. Thus, allergen sensitization should strengthen the diagnosis of asthma,[29] and interventions that reduce allergen exposure may be associated with a more favorable prognosis.[30]

Rhinitis is a risk factor for asthma in children and adults.[31–33] In children, rhinitis is frequently associated

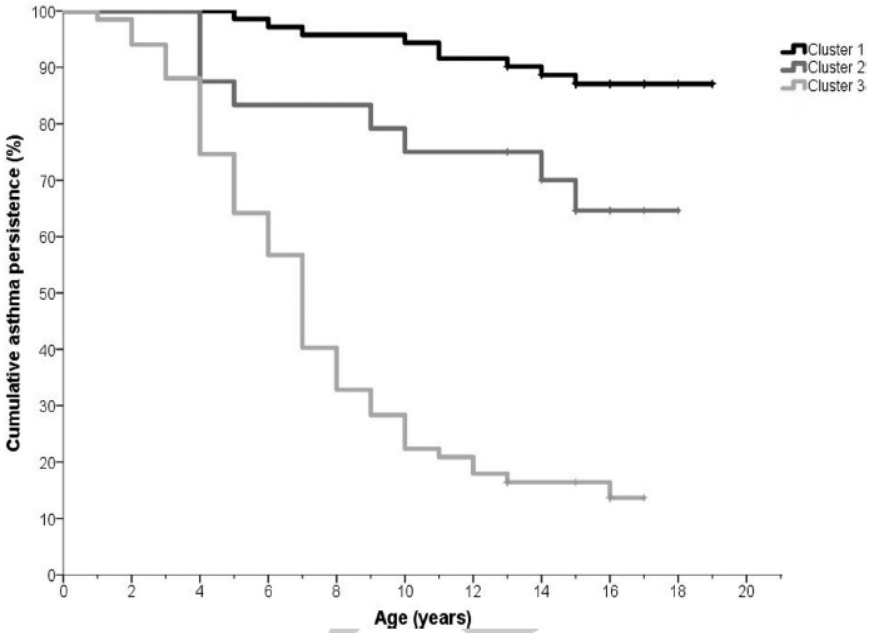

Figure 2. Cumulative asthma persistence according to age, for each defined cluster phenotype. Cluster 1 = atopic persistent wheezing; cluster 2 = nonatopic persistent wheezing; and cluster 3 = nonatopic transient wheezing

with atopy.[32] Unlike previous reports,[26–28,32] we found that, independent of atopy, rhinitis at preschool ages was a significant risk factor for asthma persistence. This finding led to the identification of the nonatopic persistent wheezing phenotype, similar to the nonatopic asthma phenotype described by the Tucson group.[17] Notwithstanding its high prevalence (estimated to be 43% in Portugal), rhinitis remains underdiagnosed, particularly in children.[34] Among preschoolers, the severity of wheezing and rhinitis are strongly associated.[35] Thus, our results emphasized the need to assess nasal symptoms in children at early ages.

Preschool eczema also was associated with asthma persistence, consistent with previous reports.[1,28,29,36] Most children with nonatopic persistent wheezing had eczema. However, we found that preschool eczema was not an independent risk factor for asthma persistence. This may have resulted from an association between eczema and rhinitis and/or atopy. The Asthma Predictive Index includes eczema as a major criterion, and rhinitis as a minor criterion for predicting disease persistence.[36] In contrast, in our population, we identified rhinitis, not eczema, as an independent risk factor for asthma persistence.

Age at enrollment was found to be associated with asthma persistence in adolescence, being a characteristic of the persistent-atopic phenotype. This was not related to age of onset of symptoms or disease duration (data not shown) and might be associated with atopy and rhinitis because these were the only identified independent risk factors.

Asthma prevalence during school ages and adolescence was higher in children with a maternal asthma history. Maternal asthma may be a risk factor for asthma persistence, which has been confirmed in other studies.[37] There was no association with paternal asthma. Although the parental asthma diagnosis was only self-reported in the present study, results of other studies indicated that maternal and paternal characteristics may have distinct impacts on offspring asthma development.[37,38]

Children who attended day care during infancy had significantly fewer persistent symptoms. However, this "protective" effect seemed to be lost with age; no effect was observed in adolescence. Also, day care attendance frequency was similar among the atopic persistent and nonatopic transient phenotypes. This indicated that the potentially protective effect of day care attendance at early ages may have depended on other characteristics of the children in each group. This hypothesis may be consistent with previous studies that demonstrated that the protective effects of day care attendance with respect to allergen sensitization and respiratory symptoms, depended on genetic variants.[39]

Exposure to maternal tobacco smoking during pregnancy is a major concern, and it has been associated with asthma in children.[31,37,40–42] Despite a significant trend over time that showed a decreasing frequency of asthma in children without maternal smoking during pregnancy, our results could not confirm that association. Similarly, we found no association between environmental tobacco exposure and asthma persistence. However, it is important to stress that, in this study, exposure to tobacco smoke was only self-reported; it was not objectively measured, and, thus, those results may be significantly biased.

Lung function abnormalities were associated with asthma persistence in adolescence. Nonatopic persistent wheezing had the worst prognosis in lung function parameters during school age and adolescence. Analysis of our results indicated that children with early wheezing onset were at risk of asthma persistence and lung function impairment when comorbidities were associated, such as rhinitis, or maternal asthma, even in the absence of atopy. Cohort studies have acknowledged atopy as a determinant of lung function,[26–28] but rhinitis alone, without atopy, has not been reported. However, a recent publication demonstrated that lung function trajectories were associated with asthma combined with comorbidities rather than with allergen sensitization.[43]

CONCLUSION

Our results supported the assignment of atopy and rhinitis at preschool ages as independent risk factors for asthma persistence in adolescence. Early wheezing in children with comorbidities, particularly rhinitis, with or without allergen sensitization, tended to predict a worse prognosis of symptom persistence and lung function in later childhood and adolescence. Our study results supported that wheezing phenotypes identified at early ages from simple measurements could predict asthma and lung function outcomes, which indicates the demand for distinct management approaches.

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Part 3: Biomarkers in allergic rhinitis and asthma multimorbidity in children

Output 5:

Lower airway patency influences peak nasal inspiratory flow in school-aged children

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Lower airway flow influences peak nasal inspiratory flow in school-aged children*

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Background: Rhinitis and asthma frequently coexist. Peak nasal inspiratory flow (PNIF) objectively evaluates nasal obstruction. Lower airway flow's impact on PNIF has seldom been analysed in children. We aimed to study the associations between PNIF and: 1)forced expiratory volume in one second (FEV_1) and peak expiratory flow (PEF) in children with allergic rhinitis and asthma and healthy controls; 2)allergic rhinitis and asthma control subjective evaluation.

Methods: Sequential assessments of PNIF before and after nasal decongestion and spirometry with bronchodilation test were performed in 65 children (6-12 years) with allergic rhinitis and asthma, and 24 gender, age-matched healthy controls. The Control of Allergic Rhinitis and Asthma Test in children (CARATkids) was used for control assessment. Associations were investigated by multiple linear regression models.

Results: Baseline and decongested PNIF correlated with baseline and post-bronchodilation FEV_1 and PEF, observed independently of rhinitis and asthma diagnosis. The best model for PNIF included PEF, age and gender. No association was found between PNIF and CARATkids scores, except for nasal obstruction self-report.

Conclusion: In school-aged children, besides age and gender, PEF values should ideally be known to interpret PNIF values. PNIF can be complementary to subjective control assessment in children with allergic rhinitis and asthma.

Key words: asthma, children, Peak Expiratory Flow (PEF), Peak Nasal Inspiratory Flow (PNIF), rhinitis

Introduction

Rhinitis and asthma frequently coexist[1-4]. Allergic rhinitis is an independent risk factor for asthma persistence[1, 5]; asthma prevalence is increased when rhinitis symptoms are persistent and severe[1, 6, 7]. Nasal obstruction is one of the main complaints in these patients[2-4, 8], due to mucosal inflammation.

The use of validated subjective scoring tools is highly recommended for the evaluation of rhinitis and asthma control[9-12]. Furthermore, the assessment of a patient suffering of nasal obstruction should be based upon subjective and objective measurements, as both may not correlate well[9, 11-13]. The Control of Allergic Rhinitis and Asthma Test (CARAT) is a simple and validated questionnaire that has the advantage of enabling the self-reported subjective concurrent assessment of rhinitis and asthma control in adults and in children (CARATkids)[14-16]. However, no data exist on the association between CARAT questionnaires and objective measures of nasal patency.

Peak nasal inspiratory flow (PNIF) is a simple and validated measure of nasal airflow[11, 12], successfully used for the objective evaluation of rhinitis and its control[17-20], including in children[21, 22]. Reference values for the paediatric population have been published, all measured in non-decongested noses[23-27]. Yet, it is important to measure PNIF values before and after decongestion to elicit the role of mucosal swelling[11, 12].

The impact of lower airway patency on PNIF should be considered[11, 12, 28-30]. A bivariate correlation between baseline PNIF and peak expiratory flow (PEF) has been described in healthy children[26], but no multivariable analysis including PEF was reported. Since a continuous increase in PNIF values has been consistently reported with children's age[23-27], it is important to exclude that

the observed association between PNIF and PEF is not a simple reflection of the normal growth (i.e., older children have larger airways and correspondingly higher nasal airflow).
Thus, we have investigated the association between PNIF before and after topical nasal alpha-adrenergic use and lower airway patency before and after inhaled $beta_2$-agonist, in children with allergic rhinitis and asthma and healthy controls, when adjusted for possible confounders. We have also evaluated the association between PNIF and CARATkids questionnaire results, multiple sensitization to aeroallergens and exhaled nitric oxide levels (FeNO).

Subjects and methods

Study design and participants

Cross-sectional, exploratory study of 65 children (6-12 years) with allergic rhinitis and asthma recruited among patients attending the Allergy Center at CUF Descobertas Hospital in Lisbon, from May until October 2015. Rhinitis was defined as the presence of nasal itching/rhinorrhea/sneezing and/or nasal obstruction, occurring during ≥2 consecutive days for >1 hour on most days[1, 8]. Allergic rhinitis was considered in children with rhinitis and self-reported symptoms upon aeroallergen exposure with positive skin prick test (SPT). Allergic rhinitis was classified according to the Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines[1, 8]. Asthma was diagnosed by a history of respiratory symptoms in the previous year, such as wheeze, breathlessness, chest tightness and cough that varied over time and in intensity, together with documented positive bronchodilator reversibility testing[10]. A convenience-driven sampling was used, stratified by asthma control defined according to the Global Initiative for Asthma (GINA) guidelines[10]; children with controlled, partly controlled and uncontrolled asthma were recruited in an approximate 1:1:1 ratio.
Children with any other diagnosed respiratory or cardiac disease were excluded from this study. These encompassed diagnosed congenital or perinatal diseases including cardiopulmonary or neuromuscular diseases, pneumonia/pneumonitis, aspiration, cystic fibrosis, immunodeficiency, gastro-oesophageal reflux disease, adenoid hypertrophy, nasal septum deviation or nasal polyps. Children with asthma exacerbation, systemic corticosteroids use or acute disease (including respiratory infection) within four weeks of the study visit were also excluded.
Age and gender-matched controls without rhinitis and asthma were screened by no positive answers on the International Study of Asthma and Allergies in Childhood questionnaire[31]. Included children were non-atopic, without history of other diseases likely to interfere with the study (such as those described above) and no acute disease within the previous four weeks.
All diagnoses were validated by medical specialists, based on anamnesis, clinical files and medical examination including anterior rhinoscopy (performed in all children).

Informed consent was obtained from each child's parent/legal guardian. This study was approved by the Ethics Committee of CUF Descobertas Hospital and by the Portuguese Data Protection Authority.

Measurements

Prior to study visit, subjects had not taken any asthma or topical nasal medication for at least 12 hours and anti-histamines for seven days. Study visits occurred during the morning.

1. CARATkids questionnaire

All children with asthma and rhinitis and their parents answered the CARATkids before all other measurements[15]. Total score (0 – best control to 13 – worst control) was recorded, as well as, nasal obstruction report by the children and CARATkids total nasal score (varying from 0 to 3, obtained by the sum of positive answers regarding nasal symptoms of "blocked nose", "sneezing" and "runny nose", in the past two weeks).

2. Anthropometric measurements

Accurate height (cm) and weight (Kg) were registered without shoes for all the included children.

3. Respiratory function tests

All nasal and lung function tests were performed by trained lung function technicians, all awarded with the European Respiratory Society Spirometry Driving Licence.
Bilateral PNIF was assessed using a nasal flow meter (In-Check Nasal, Clement Clarke International Ltd., Edinburgh, England). After mildly blowing their noses, the children were instructed to do a nasal inspiration with their mouths closed, from residual volume to total lung capacity, while using the facial mask without leakage of air or nose compression. The highest value out of three satisfactory maximal inspiration measurements was recorded. All measurements were taken with children sitting in an upright position.
Lower airway flow was assessed by forced expiratory volume in one second (FEV_1) and PEF, obtained by spirometry test (Jaeger MasterScreen™ system from Carefusion with a Flow Spirometer (Lilly Pneumotacograph) with Software version - JLAB 5.31.0.83), performed and interpreted according to international recommendations[32]. Global Lung Initiative normative data was used. Bilateral topical nasal phenylephrine chlorhydrate at 2.5mg/ml was applied and a bronchodilation test was performed with 400µg of inhaled salbutamol administered using a chamber[32]. Then, decongested PNIF was obtained using the same procedures, as well as post-bronchodilator PEF and FEV_1.
Nasal reversibility was calculated by the Nasal Congestion Index (NCI)[33], as follows: NCI = [(decongested PNIF – baseline PNIF) / baseline PNIF]x100.
For the PNIF measurements recorded, the z-scores for age and

Table 1. Study group characteristics.

Characteristic	All study participants (n=89)	Healthy controls (n=24)	Controlled asthma (n=20)	Partly controlled asthma (n=21)	Uncontrolled asthma (n=24)	p
Gender male, n (%)	50 (56.2)	12 (50.0)	10 (50.0)	15 (71.4)	13 (54.2)	0.439
Age months, median (min-max)	115 (73.0-155.0)	116 (73.0-155.0)	105 (74.0-155.0)	115 (76.0-148.0)	128 (78.0-155.0)	0.106
Height cm, median (min-max)	135 (113.0-165.0)	133 (114.5-155.0)	134 (116.0-150.5)	134 (113.0-162.3)	143 (117.0-165.0)	0.154
Weight Kg, median (min-max)	34 (17.0-67.0)	31 (17.0-48.0)	31 (20.0-53.0)	35 (17.0-54.0)	39 (22.0-67.0)	0.103
Allergic rhinitis classification (ARIA)*						0.028
Mild intermittent, n (%)	5 (5.6)	0 (0.0)	4 (20.0)	1 (4.8)	0 (0.0)	
Moderate-severe intermittent, n (%)	2 (2.3)	0 (0.0)	0 (0.0)	2 (9.5)	0 (0.0)	
Mild persistent, n (%)	11 (12.4)	0 (0.0)	5 (25.0)	3 (14.3)	3 (12.5)	
Moderate-severe persistent, n (%)	47 (52.8)	0 (0.0)	11 (55.0)	15 (71.4)	21 (87.5)	
Nasal topic corticosteroid use*#, n (%)	23 (33.8)	0 (0.0)	6 (30.0)	8 (38.1)	9 (37.5)	0.832
Oral montelukast use*#, n (%)	7 (7.9)	0 (0.0)	0 (0.0)	3 (14.3)	4 (16.7)	0.201
Inhaled corticosteroid use*#, n (%)	37 (41.6)	0 (0.0)	12 (60.0)	14 (66.7)	11 (45.8)	0.351
Inhaled LABA use*#, n (%)	20 (22.5)	0 (0.0)	6 (30.0)	4 (19.0)	10 (41.7)	0.259
Multiple sensitization*, n (%)	39 (60.0)	0 (0.0)	11 (55.0)	13 (61.9)	15 (62.5)	0.860

ARIA – allergic rhinitis and its impact on asthma guidelines; min – minimum; max – maximum; LABA – long-acting beta$_2$ agonist; * - considering only children with rhinitis and asthma, # – medication use reported by caregiver

gender were calculated according to the PNIF reference values proposed by Papachristou and collaborators[24].

4. Fractional exhaled nitric oxide measurement

The FeNO was measured with the NIOX Vero® system (Aerocrine, Sweden) using a single-breathing online method before respiratory function tests, according to current guidelines[34, 35].

5. Skin prick tests

The SPT were performed according to international recommendations[9, 36]. The skin was pricked with steel lancets (Prick Lancetter®, Hollister-Stier Laboratories, Spokane, WA, USA), and the following allergen extracts were applied: *Dermatophagoides pteronyssinus, Dermatophagoides farinae, Lepidoglyphus destructor, Parietaria*, grass pollen mixture, olive tree, *Alternaria*, dog and cat epithelium (Bial-Aristegui®, Bilbao, Spain). Histamine hydrochloride (10 mg/ml) was used as a positive control; a saline solution was the negative control. A wheal mean diameter ≥3 mm was considered a positive test.

Atopy was considered when at least one SPT was positive. Multiple sensitization was defined when SPT were positive to two or more of the following aeroallergen groups: house dust mites, pollens, moulds or pets.

Statistical analysis

Normal distribution of variables was assessed using the Shapiro-Wilk or Kolmogorov-Smirnov tests and histogram visual analysis. Categorical variables were expressed as absolute and relative frequencies. Comparisons were performed with the Pearson's chi-square test. Continuous variables were expressed as the mean and standard deviation (SD), or median and extreme values in case of no normal distribution. Comparisons were performed with parametric independent t-test or one-way ANOVA or non-parametric Mann-Whitney or Kruskal-Wallis tests, followed by post-hoc multiple comparisons according to Bonferroni test. Correlations were analysed using Pearson's coefficient or Spearman's rank coefficient. Associations were further investigated by multivariable linear regression models for baseline and decongested PNIF; assumption of normally distributed residuals was fulfilled. Results were reported as beta-coefficients with 95% confidence intervals (95%CI).

P values <0.05 were considered significant. Data analyses were performed with SPSS® version 24.0 for Windows (IBM SPSS, Chicago, IL, USA).

Results

Participants characteristics

A total of 89 children were included. Participants' characteristics are summarized in Table 1. All children were Caucasian. No statistically significant differences were found regarding demographic and anthropometric parameters. Most patients had moderate-severe persistent allergic rhinitis (72.3%). Allergic rhinitis classification by ARIA was associated with asthma control assessed according to GINA guidelines (Table 1).

Table 2. Control of Allergic Rhinitis and Asthma Test for children (CARATkids) results.

CARATkids	Total of asthmatic children (n=65)		Controlled asthma (n=20)		Partly controlled asthma (n=21)		Uncontrolled asthma (n=24)		p
Global score, median (min-max)	4	(0-10)	1	(0-8)	4	(0-10)	6	(2-10)	<0.001
Nasal obstruction, n (%)	38	(58.5)	9	(45.0)	12	(57.1)	17	(70.8)	0.221
Nasal score, n (%)									0.121
0	10	(15.4)	4	(20.0)	4	(19.0)	2	(8.3)	
1	12	(18.5)	7	(35.0)	4	(19.0)	1	(4.2)	
2	22	(33.8)	4	(20.0)	7	(33.3)	11	(45.8)	
3	21	(32.3)	5	(25.0)	6	(28.6)	10	(41.7)	

min – minimum; max – maximum

Table 3. Study group objective airway function and exhaled nitric oxide assessment.

Characteristic	All study participants (n=89)		Healthy controls (n=24)		Controlled asthma (n=20)		Partly controlled asthma (n=21)		Uncontrolled asthma (n=24)		p
Baseline PNIF L/min, median (min-max)	100.0	(40.0-170.0)	100.0	(60.0-150.0)	100.0	(40.0-150.0)	100.0	(50.0-150.0)	100.0	(40.0-170.0)	0.747
Decongested PNIF L/min, median (min-max)	110.0	(50.0-210.0)	110.0	(80.0-190.0)	110	(50.0-200.0)	120.0	(60.0-190.0)	120.0	(80.0-210.0)	0.339
Baseline PNIF z-score, mean (SD)	-0.77	(0.94)	-0.70	(0.94)	-0.80	(1.06)	-0.65	(0.66)	-0.93	(1.06)	0.630
Decongested PNIF z-score, mean (SD)	-0.07	(0.93)	-0.05	(1.05)	-0.16	(1.00)	0.12	(0.82)	-0.17	(0.87)	0.617
Nasal congestion index %, median (min-max)	21.43	(-10.00-175.00)	23.61	(0.00-66.67)	19.26	(-10.00-100.00)	20.00	(-10.00-83.33)	22.48	(-4.55-175.00)	0.984
Baseline PEF L/s, mean (SD)	3.95	(0.97)	3.96	(0.87)	3.98	(0.96)	3.98	(1.26)	3.90	(0.83)	0.993
Baseline PEF z-score, mean (SD)	-0.41	(0.94)	-0.25	(0.79)	-0.07	(1.08)	-0.49	(0.85)	-0.77	(0.96)	0.070
Post-beta$_2$ agonist PEF L/s, mean (SD)	4.30	(0.99)	4.28	(0.83)	4.27	(1.02)	4.17	(1.25)	4.47	(0.87)	0.776
Post-beta$_2$ agonist PEF z-score, mean (SD)	0.01	(0.81)	0.21	(0.77)	0.22	(0.92)	-0.20	(0.91)	-0.18	(0.59)	0.135
Baseline FEV$_1$ L, mean (SD)	1.88	(0.47)	1.91	(0.48)	1.93	(0.47)	1.86	(0.57)	1.83	(1.89)	0.894
Baseline FEV$_1$ z-score, mean (SD)	0.32	(1.58)	0.85	(1.07)	1.26	(1.65)	0.19	(1.30)	-0.88	(1.42)	<0.001
Post-beta$_2$ agonist FEV$_1$ L, mean (SD)	2.05	(0.51)	1.98	(0.47)	2.04	(0.53)	2.01	(0.59)	2.15	(0.48)	0.698
Post-beta$_2$ agonist FEV$_1$ z-score, mean (SD)	1.22	(1.35)	1.30	(1.09)	1.79	(1.47)	1.11	(1.35)	0.75	(1.36)	0.079
Positive bronchodilation test, n (%)	25	(28.1)	1	(4.2)	0	(0.0)	6	(28.6)	18	(75.0)	<0.001
FeNO ppb, median (min-max)	12	(0-104)	5	(0-24)	10	(0-87)	15	(0-94)	29	(7-104)	<0.001

PNIF – peak nasal inspiratory flow; PEF – peak expiratory flow; FEV$_1$ – forced expiratory volume in one second; FeNO – exhaled nitric oxide; min – minimum; max – maximum; SD – standard deviation

Table 4. Correlation coefficients between Peak Nasal Inspiratory Flow and demographic/anthropometric and lung function variables.

Variables	Baseline PNIF (L/min)		Decongested PNIF (L/min)	
	rho	p	rho	p
Age (months)	0.281	(0.008)	0.445	(<0.001)
Height (cm)	0.288	(0.006)	0.452	(<0.001)
Weight (Kg)	0.358	(0.001)	0.488	(<0.001)
Baseline FEV_1 (L)	0.322	(0.002)	0.453	(<0.001)
Baseline FEV_1 (z-score)	-	ns	-	ns
Post-$beta_2$ agonist FEV_1 (L)	0.299	(0.004)	0.475	(<0.001)
Post-$beta_2$ agonist FEV_1 (z-score)	-	ns	-	ns
Baseline PEF (L/s)	0.404	(<0.001)	0.482	(<0.001)
Baseline PEF (z-score)	0.321	(0.002)	-	ns
Post-$beta_2$ agonist PEF (L/s)	0.341	(0.001)	0.476	(<0.001)
Post-$beta_2$ agonist PEF (z-score)	0.226	(0.033)	-	ns

rho – Spearman rank correlation coefficient; PNIF – peak nasal inspiratory flow; FEV_1 – forced expiratory volume in one second; PEF – peak expiratory flow; ns – not significant

CARATkids scores

The median CARATkids score in children with rhinitis and asthma was 4, varying from 0 to 10. This score was associated with asthma control but no statistically significant differences were found regarding CARATkids nasal score or nasal obstruction self-report (Table 2).

The CARATkids score was associated with ARIA rhinitis classification, the worst values in moderate-severe persistent rhinitis (Bonferroni post-hoc testing, p=0.002).

Objective measurements

Table 3 summarizes the upper and lower airway function and FeNO assessment results. No statistically significant differences were found in PNIF values or NCI comparing healthy children versus children with rhinitis and asthma, either reporting nasal obstruction or not. Children with rhinitis and asthma reporting nasal obstruction in the past two weeks in the CARATkids questionnaire had mean baseline PNIF z-scores of -1.02 (SD 1.01) versus -0.68 (SD 0.95) in healthy children (p=0.185).

Obstructive lower airway flow limitation was found in 27% of the children, with FEV_1 z-score varying from -3.49 to 1.17. Lower FEV_1 z-score and higher FeNO levels were found in children with asthma, the worst values in uncontrolled asthmatics (Bonferroni post-hoc testing, p≤0.006). No associations were found between PNIF and asthma control (assessed according to GINA guidelines) or ARIA classification of allergic rhinitis.

Association between PNIF and CARATkids scores

In children with rhinitis and asthma, those reporting nasal obstruction in the CARATkids questionnaire had lower baseline PNIF z-scores (mean -1.02 (SD 1.01) versus mean -0.49 (SD 0.73), p=0.018). This association was independent of PEF and FEV_1. No other associations were found between PNIF and CARATkids scores.

Association between PNIF and demographic/anthropometric variables

Baseline and decongested PNIF values correlated with age, height and weight (Table 4). Both values were higher in boys, although this difference was not statistically significant (median decongested PNIF 120.0L/min (70.0L/min-210.0L/min) in boys versus 110.0L/min (50.0L/min-190.0L/min) in girls, p=0.117).

Association between PNIF, lung function variables and FeNO

Baseline and decongested PNIF correlated with baseline and post-$beta_2$ agonist FEV_1 and PEF (Table 4). After adjusting the models for gender and age, the estimated change in decongested PNIF per 1L increase in post-$beta_2$ FEV_1 or 1L/s increase in PEF (beta-coefficients) were 29.73L/min (95%CI 17.80-41.67, adjusted r^2=0.248, p<0.001) and 17.12L/min (95%CI 11.06-23.19, adjusted r^2=0.293, p<0.001), respectively. Baseline values of PNIF were also significantly associated with FEV_1 (beta-coefficient 15.78L/min; 95%CI 3.92-27.64, adjusted r^2=0.105, p=0.008) and PEF (beta-coefficient 10.40L/min; 95%CI 4.77-16.02, adjusted

r^2=0.164, p<0.001).
The backward elimination procedure eliminated the variables height and weight. The associations between PNIF and FEV_1 or PEF were observed independently of rhinitis and asthma diagnosis, CARATkids scores, multiple sensitization and montelukast, nasal or inhaled corticosteroid or long-acting $beta_2$-agonist use. The best equation for decongested PNIF (L/min) was (adjusted r^2=0.309, p<0.001): 30.32 +11.38 x baseline PEF (L/s)+0.34 x age (months) +14.89 x gender [0–female; 1-male].

A correlation was found between baseline PNIF (L/min) and PEF z-score (Table 4). Baseline PNIF z-score was also associated with FEV_1 z-score (r=0.228, p=0.031) and PEF z-score (r=0.307, p=0.003); this was observed independently of nasal obstruction report.There was no association between reversibility of the upper and lower airways. No association was found between PNIF and FeNO levels.

Discussion

A consistent and independent correlation between PNIF and lower airway patency was found in school-aged children. Thus, this study strengthens that, ideally, it is important to consider at least PEF values, besides age and gender, when assessing nasal airflow by means of PNIF in children. PNIF can provide complementary objective information to subjective control assessment in children with allergic rhinitis and asthma.
We have evaluated the association between PNIF and lower airway patency measures in school-aged children, when adjusted to potential confounders. Sequential upper and lower airway standardized evaluations of the same individual were performed, reporting PNIF values before and after nasal topical alpha-adrenergic use and PEF and FEV_1 values before and after inhaled $beta_2$-agonist. A balanced sample of patients comprising different asthma control levels was studied, together with age and gender-matched healthy controls. Consistent correlations between upper and lower airway flow were found. These correlations persisted after adjusting to age, gender, height and weight, suggesting that the association between upper and lower airflow measures is independent and not a simple reflection of growth or body size. The correlations found between PNIF and PEF expressed in z-scores (which is age, gender and height-independent) also support our conclusions. Furthermore, this study strengthens that objective measurements of nasal flow and subjective symptoms scores may not be correlated, as we found no associations between PNIF and the CARATkids questionnaire scores, except for nasal obstruction self-report in the past two weeks and PNIF expressed in z-scores in children with allergic rhinitis and asthma.
This study's main limitations are its cross-sectional, exploratory design and the lack of children with asthma or rhinitis alone. Most children with asthma also have rhinitis[1, 5, 9, 10], and we did include asthmatic children with different levels of disease control and lung function tests results, but still inclusion of children with asthma or rhinitis only is relevant. Another limitation is the lack of children of other ethnicity, which limits our results only to Caucasians. Further studies with larger heterogeneous samples are needed to confirm our results. Despite the fact that all diagnoses were validated by medical specialists (based on anamnesis, clinical files and medical examination including anterior rhinoscopy), no nasal endoscopy or imaging techniques were performed in this study. Therefore, we could not firmly rule out adenoid hypertrophy or nasal septum deviation, but only excluded children with known or suspected diagnosis or with observable anatomical nasal abnormality on anterior rhinoscopy. Although baseline PNIF correlated with PEF and FEV_1 before and after bronchodilator use, we did not retest for PNIF after the single use of bronchodilator and therefore the clinical usefulness of PNIF after bronchodilator only was not tested.
The fact that nasal flow can be influenced by lower airway status has long been a concern[12]. Studies in adults have suggested an independent correlation between PNIF and PEF[28], or FEV_1 [29]. Although initially considered a limitation of PNIF, the concept of a single disease of the airways has changed this view, and the impact of the lower airways is now taken into consideration in the study of nasal function[12, 37]. The correlation between PNIF and PEF has been reported in healthy children[26], but no multivariable analysis including PEF values was reported and FEV_1 wasn't evaluated. As previously described[24-26], we have found a correlation between PNIF and age, height and weight. PNIF values were higher in boys compared to girls but, as reported by Papachristou et al., this difference did not reach statistical significance until the age of 12 years[24]. Since age was the only variable consistently reported in the literature to be correlated with PNIF[23-27], multivariable linear models for PNIF using this variable were presented. We chose to include baseline PEF rather than FEV_1 in the final model since baseline PEF is more easily obtained, even with only a peak flow meter and without the need to use bronchodilators. After accounting for these variables, there still remains a large degree of variability in PNIF values, suggesting that further variables, such as anatomic variations or possibly nasal inflammation may refine the modelling of data. Using acoustic rhinometry (before and after alpha-agonist) and spirometry with bronchodilation test, Chawes and collaborators found an independent and consistent correlation between nasal volume and FEV_1 in children aged six years[38]. In accordance to our results, the correlation between upper and lower airways measures were observed independently of rhinitis and asthma diagnosis[38]. This suggests that the independent correlation is a consistent finding in healthy and asthmatic children. Our results further support the hypothesis that the correlation between upper and lower airways may reflect a physiologic background for the common asthma-rhinitis multimorbidity[38].

Pathologic mechanisms could also be involved. A continuous nasobronchial inflammation process has been described in rhinitis and asthma[9, 10]. However, we found no association between PNIF and FeNO levels or between reversibility of the upper and lower airways, also in agreement with Chawes et al findings[38]. We also found no differences in PNIF according to multiple sensitization to aeroallergens, as described before[38, 39]. Nevertheless, Chawes and collaborators reported an association between blood eosinophil counts and nasal eosinophilia with nasal patency[38], which we did not evaluate.

Our baseline PNIF values were similar to the reported values in healthy children in Brazil[25, 26]. Prescott et al. and van Spronsen et al. reported slightly lower PNIF values[23, 27], while Papachristou et al., who analysed the largest sample of healthy children, reported higher PNIF values[24]. The discrepancy between study results could be due to different studied populations and to different methods (for instance, not performing the PNIF manoeuvre from the residual volume until reaching total lung capacity[23], or PNIF values collected while sitting[23, 27], or standing up[24-26]). A recent study in adults showed a trend towards a positive effect of the standing position on PNIF, although not statistically significant[40]. To our knowledge, the effect of body position on PNIF in children has not been analysed. Papachristou et al. published PNIF values were similar to our observed decongested PNIF values[24], which we used as a reference.

In our study, no association was found between PNIF values and GINA asthma control or ARIA rhinitis classifications, or with CARATkids total or nasal scores. Previous studies with children have found an agreement between objective and subjective measures of nasal obstruction[21, 41], while others report the opposite[42]. The reason for this disagreement in multiple studies is probably multifactorial. For instance, given the known influence of other variables including age, gender and anthropometric variables in upper airway function in children, it is important to use reference values to obtain predicted percentages or ideally z-scores to interpret the results. We found an association between PNIF expressed in z-scores (for age and gender) and nasal obstruction self-report in the CARATkids questionnaire in children with allergic rhinitis and asthma. This association was also independent of PEF. Evaluating each nostril individually instead of bilateral measurements has been shown to allow stronger correlations between objective and subjective measurements in adults[13], and also described in children using rhinomanometry[42]. On the other hand, the subjective scoring tool influences the results. Scales with fewer score options seem to increase the probability of an association between objective and subjective measurements[43]. The exact questions and time of symptoms evaluation may also affect this association. In our study, we didn't find statistically significant differences in PNIF comparing healthy children and children with rhinitis and asthma reporting nasal obstruction in the CARATkids questionnaire. This needs to be analysed in future studies with larger samples, but it could be influenced by the fact that CARATkids questionnaire considers nasal symptoms self-reported by the children regarding the last two weeks and not necessarily current symptoms at the time of PNIF measurement. Moreover, in a previous study in adults, patients with asthma significantly rated their nasal obstruction by visual analogue scale more seriously than non-asthmatic controls with comparable PNIF values[29]. Apparently, the sensation of nasal obstruction in asthmatics may be different from controls despite being in the same PNIF group[29]. On the opposite direction, children on long-term treatment for chronic rhinitis may underreport the amount of nasal congestion[44], and it has been reported that children may be more accepting of mouth-breathing than adolescents[43]. Last but definitely not least, children might also be influenced by their parents or guardians' perceptions during the subjective assessments, which may also contribute to this disagreement[42]. Therefore, as in asthma, objective and subjective assessments appear to evaluate different parameters that may not be directly related, and PNIF may provide complementary information to the subjective evaluation of rhinitis and asthma in children. Since the subjective feeling of nasal obstruction may be valued differently by individual subjects, future research studies addressing PNIF in children with rhinitis and asthma may take advantage of additional comparisons with other measures of nasal patency, namely using the "golden standard" rhinomanometry.

Conclusion

In conclusion, the independent correlation between PNIF and lower airways patency measures in school-aged children suggests that, ideally, at least PEF values should be considered, besides age and gender, when evaluating nasal obstruction by means of PNIF in this age group. This study supports PNIF as a complementary objective measurement to the subjective assessment of allergic rhinitis and asthma control in children.

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Authorship contribution

HP participated in study conception, data collection, analysis and interpretation and wrote the manuscript draft. LP, ACH, IM, CC, AVL and IA participated in data collection. MB provided critical review. MMA critically analysed the project, with guidance throughout its stages and tasks. All authors have reviewed and approved the final manuscript.

Conflict of interest

None.

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Exploratory salivary and urinary metabolomics of childhood allergic rhinitis and asthma multimorbidity

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Full title: Exploratory salivary and urinary metabolomics of childhood allergic rhinitis and asthma multimorbidity

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ABSTRACT

Background: Exploratory molecular profiling of asthma phenotypes may contribute with novel insights to incorporate pathobiologic characteristics and biomarkers in the progression from clinical phenotypes to endotypes. This study aimed to uncover “hypothesis-free” salivary and urinary differentiating metabolic features in children with allergic rhinitis and asthma multimorbidity compared to healthy children.

Methods: Cross-sectional exploratory study including 65 children (6-12 years) with allergic rhinitis and asthma and 24 healthy children, matched for age and gender. All children underwent saliva and urine collection, spirometry with bronchodilation test, exhaled nitric oxide (FeNO) measurement and skin prick tests. Untargeted metabolomics analysis of saliva and urine was performed using nuclear magnetic resonance (NMR) spectroscopy. Spectroscopic and clinical data were subjected to multivariable and univariable statistical analyses.

Results: Principal component analysis showed no sample clustering according to allergic rhinitis and asthma in the salivary and urinary metabolic profiles. Univariable analysis followed by linear modelling revealed subsets of NMR spectral areas significantly different in the children with allergic rhinitis and asthma. Some metabolites contributing to these variables were identified: arginine, taurine, citrate and aspartate (in saliva), and quinolinate, butyrate, pantothenate, gluconate, pseudouridine and lysine (in urine). Quinolinate, butyrate and pantothenate levels correlated with spirometric parameters, while quinolinate, gluconate and pseudouridine correlated with FeNO levels. Urinary quinolinate and salivary citrate and aspartate levels were associated with multiple allergenic sensitization.

Conclusion: The NMR-based exploratory metabolomics revealed differentiating subsets of spectral features in saliva and urine in children with allergic rhinitis and asthma multimorbidity, compared to healthy children. Some of these spectral areas were associated with lung function parameters, FeNO levels and multiple allergenic sensitization. Identified metabolites contributing to these areas included the previously reported asthma-related metabolites arginine, taurine, quinolinate and butyrate, and may support further studies regarding their role in allergic rhinitis and asthma multimorbidity-related metabolic pathways.

Keywords: Allergy, Asthma, Children, Metabolomics, Multimorbidity, Nuclear Magnetic Resonance, Phenotype, Rhinitis.

INTRODUCTION

Worldwide since early childhood, asthma affects the lives of several hundred million people. In spite of the advances in the last decades, this chronic disease remains associated with significant morbidity and high costs, mostly due to uncontrolled asthma[1, 2]. The complex heterogeneity and dynamics of the asthma syndrome is driving the search for distinct asthma clinical phenotypes, aiming further to look for asthma endotypes, i.e., "subtypes of asthma", each being caused by distinct underlying pathophysiological mechanisms, with different treatment and prognosis, that would allow more targeted, effective (novel) therapeutic approaches[3, 4]. Presently, no widely agreed criteria upon asthma endotypes are described. Exploratory metabolic profiling of asthma can enable the detection of "hypothesis-free" biomarkers of the disease at the molecular level and provide novel insights on pathophysiologic mechanisms to the progression from clinical phenotypes to endotypes.

Data from pulmonary tissue may hold the best chance of obtaining relevant data for specific asthma endotypes, given the direct airways evaluation. However, biomarkers need to be developed in accessible compartments that can be analyzed relatively easily and repeatedly. Urine is easily collected in most clinical scenarios and it is metabolically interesting as an end-product sampling of metabolic activity of a given organism[5]. Preliminary metabolomics data have shown that urine samples may be a surrogate of pathophysiologic processes that occur in asthma and suggested distinct metabolic features associated with this disease[6-14]. Recently, liquid chromatography combined with mass spectrometry was applied to a pilot study of saliva samples in asthmatics, also demonstrating the potential to discriminate asthma[15]. To date, untargeted metabolomics studies have reported a broad number of different metabolites associated with asthma[16, 17]. Some metabolites were identified as significant in at least two exploratory studies, including adenosine, arginine, phenylalanine, taurine, tyrosine, glucose, acetate, butyrate, propionate, formate, ethanol, methanol and urocanate[8, 9, 18-25]. One possible explanation for non-replicated results may be associated with the fact that most of the previous metabolomics studies focused on distinguishing asthma cases from healthy controls, with only a very few number of studies examining particular clinical asthma phenotypes[16, 17]. Exploring the metabolic profiling of clinically defined asthma phenotypes reduces heterogeneity and potential bias. The most prevalent and consensual phenotype in childhood is "allergic asthma", typically accompanied by clinically relevant sensitization to allergens and association with other diseases, in particular allergic rhinitis[4]. The well-known link between allergic rhinitis and asthma has recently been supported by large-scale unbiased clustering at the population level, suggesting that undisclosed mechanisms underlying these diseases need to be investigated together, considering the childhood "allergic multimorbidity" cluster[26, 27].

In this study, we hypothesized that the metabolic activity of children with allergic rhinitis and asthma would differ from that of healthy children and that NMR could measure such differences in saliva and urine samples. Thus, we aimed to uncover "hypothesis-free" differences in salivary and urinary metabolic features between school-aged children with and without allergic rhinitis and asthma multimorbidity.

MATERIALS AND METHODS

Study design and participants

In total, 65 children (aged 6-12 years) with allergic rhinitis and asthma were recruited for a cross-sectional study in the Allergy Center outpatient's clinic at CUF Descobertas Hospital in Lisbon, from May until October 2015.

Asthma was diagnosed according to the Global INitiative for Asthma (GINA) recommendations[4], by a history of respiratory symptoms such as wheeze, breathlessness, chest tightness and cough that varied over time and in intensity, occurring for more than 12 months, together with variable expiratory airflow limitation, documented by positive bronchodilator reversibility testing. Rhinitis was defined as the presence of nasal itching/rhinorrhea/sneezing and/or nasal obstruction, occurring during at least two consecutive days for more than one hour on most days[28, 29]. Allergic rhinitis and asthma multimorbidity was considered in those children with self-reported symptoms of rhinitis and asthma upon aeroallergen(s) exposure, with positive skin prick test to the allergen(s). A convenience-driven sampling was used, stratified according to asthma control defined by current GINA recommendations[4]; children with controlled, partly controlled and uncontrolled asthma were recruited in an approximate 1:1:1 ratio.

Children with any other diagnosed inflammatory (except atopic dermatitis[30]), respiratory or cardiac disease were excluded. These encompassed diagnosed congenital or perinatal diseases including cardiopulmonary diseases, pneumonia / pneumonitis, aspiration, cystic fibrosis, immunodeficiency or gastro-esophageal reflux disease. Children with asthma exacerbation, systemic corticosteroids use or respiratory infection within four weeks of the study visit were also excluded.

Age and gender-matched healthy children were screened by no positive answers on the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire[31], and no atopy assessed by skin prick tests. These children had no history of respiratory, inflammatory or other diseases likely to interfere with the study (such as those described above) and no acute disease within the previous four weeks or any drug intake within the previous eight days before the study visit.

All diagnoses were validated by medical specialists, based on anamnesis, clinical files and medical examination (performed in all children).

Informed consent was obtained from each child's parents/legal guardians. This study was approved by the Ethics Committee of CUF Descobertas Hospital and of NOVA Medical School and by the Portuguese Data Protection Authority.

Sample collection and Measurements

Prior to the study visit, subjects had not taken inhaled corticosteroids, short-acting beta-agonists and anti-cholinergics for >12 hours; long-acting bronchodilators for >24 hours, leukotriene receptors antagonists for >36 hours, and anti-histamines for >7 days. All children were asked to refrain from any drink, food intake or tooth brushing at least one hour before samples collection.

In the study visit, saliva and urine samples were consecutively collected, followed by exhaled nitric oxide (FeNO) measurement and, thereafter, lung function and skin prick tests. Each child performed all tests at the hospital in the same study visit, which occurred during the morning.

1. Saliva and urine samples collection

Saliva was collected by a passive drool technique into a sterile cup cooled on ice. Immediately after, midstream urine specimens were collected into sterile cups. All samples were immediately stored (-80ºC) until analysis.

2. NMR measurements

All chemicals and consumables were purchased from Sigma-Aldrich® (Germany) except for the NMR tubes and 96-well Ritter plates, which were purchased from Bruker® (Bruker Biospin Ltd., USA). Frozen urine and saliva aliquots were thawed in a refrigerator overnight. Cellular components and other insoluble material were spun down by centrifugation for 10min at 3184×g and 4°C. The supernatants were transferred into the 96 Ritter plates. A volume of 0.225mL of each supernatant was mixed with 0.025mL of phosphate buffer (1.5M K_2HPO_4/KH_2PO_4, 2mM NaN_3 in D_2O, pH 7.4) containing 4mM of sodium 3-trimethylsilyl [2,2,3,3-D_4] propionate as internal standard and chemical shift reference. A volume of 0.165mL of each sample-buffer mixture was transferred to 3mm SampleJet NMR tubes using a Gilson 215 robotic liquid handler and placed in refrigerated racks (6°C) of a SampleJet system (Bruke Biospin Ltd., USA) until the NMR measurements.

NMR spectra were collected on a Bruker 14.1T AVANCE II spectrometer, equipped with a triple resonance inverse cryoprobe. For each sample a one-dimensional (1D) ^{1}H NMR experiment was collected at 27˚C, using the noesygppr1d pulse program (Topspin 3.0, Bruker Biospin Ltd., USA)[32]. Excitation pulses were automatically calibrated for each sample, while the water signal was suppressed by presaturation. The recorded data was automatically processed (phase and baseline correction, line broadening by a factor of 1Hz and referencing to the chemical shift of 3-trimethylsilyl [2,2,3,3-D_4] propionate at 0.0ppm). All NMR measurements were performed in a random order.

Two dimensional (2D) ^{1}H J-resolved (Jres) NMR spectra were also recorded for each sample to assist the assignment of NMR peaks. Moreover, the homonuclear (^{1}H-^{1}H) Correlation Spectroscopy and Total Correlation Spectroscopy as well as the ^{1}H-^{13}C Heteronuclear Single-Quantum Correlation experiments were acquired for a small set of pooled samples. These samples were made by mixing small aliquots from each of the samples of the study either for urine or saliva. The 2D data was exclusively used for the identification of the NMR peaks in combination with the proprietary database Biorefcode implemented in AMIX software (Bruker Biospin Ltd., USA).

3. Fractional exhaled nitric oxide measurement

The FeNO was measured with the NIOX Vero® system (Aerocrine, Sweden) using a single-breathing online method, according to current guidelines[33, 34].

4. Lung function tests

Spirometry with bronchodilation test (Jaeger MasterScreen™ system from Carefusion with a Flow Spirometer (Lilly Pneumotacograph) with Software version - JLAB 5.31.0.83) were performed and interpreted, according to international recommendations[35]. Global Lung Initiative normative data was used, reporting children's lung function based on z-scores.

5. Skin prick tests

Skin prick tests were performed according to international recommendations[36]. The skin was pricked with steel lancets (Prick Lancetter®, *Hollister-Stier Laboratories*, USA), and the following allergen extracts were applied: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Lepidoglyphus destructor, Parietaria*, grass pollen mixture, olive tree pollen, *Alternaria,* dog and cat epithelium (*Bial-Aristegui®,* Spain). Histamine hydrochloride (10 mg/ml) was used as a positive control; a saline solution was the negative control. A wheal mean diameter >3 mm was considered a positive test.

Atopy was considered when at least one skin prick test was positive. Multiple sensitization was defined when skin prick tests were positive to two or more of the following aeroallergen groups: house dust mites, pollens, molds and pets.

Statistical analysis

1. Analysis of clinical and demographic data

Continuous variables were expressed as the mean and standard deviation, or median and extreme values in case of no normal distribution. Comparisons were performed with parametric independent t-test or one-way ANOVA or non-parametric Mann-Whitney or Kruskal-Wallis tests, followed by post-hoc multiple comparisons according to Bonferroni test. Correlations were analyzed using Spearman's rank coefficient. Categorical variables were expressed as absolute and relative frequencies. Comparisons were performed with the Pearson's chi-square test. P-values <0.05 were considered significant. Data analyses were performed with SPSS® version 24.0 (IBM SPSS, Chicago, IL, USA).

2. Analysis of spectroscopic data

Pre-processing of NMR data prior to statistical analysis was performed with in house routines written in Matlab 2014a (The Mathworks, Inc., USA) and Python 2.7 (Python Software Foundation, www.python.org, USA). The region from 0.5 to 9.5ppm of all 1D NMR spectra was binned using the adaptive intelligent binning approach[37], resulting in 381 variables (bins) for urine and 450 variables for saliva. Both datasets were normalized using the Probabilistic Quotient Normalization method to correct for dilution differences[38]. The final dataset consisted of 381 variables x 89 samples for urine and 450 variables x 88 samples for saliva (the spectrum from one sample did not meet the quality criteria for further analysis and was excluded). Finally, the normalized data was centered, scaled to unit variance and log transformed for the statistical analysis.

The statistical analysis was performed in R statistical environment (http://www.r-project.org/, R versions 3.3.2, Austria). Two main statistical approaches were used. In the first approach, multivariable analysis was performed with Principal Component Analysis (PCA). In the second approach, Mann-Whitney U test was used for variable preselection with regard to the differences between children with allergic rhinitis and asthma (with partly controlled or uncontrolled asthma) and healthy children (significance was expressed at $p<0.05$). Further subset selection was performed by linear modelling. Stepwise linear regression was applied to select the final best set of variables (Figure 1).

Identification of the metabolites contributing to the significant variables (odds-ratio ≠1) was performed by search of the Biorefcode (Bruker Biospin Ltd., USA) database and further verification of the annotated metabolites was done by analysis of the collected 2D NMR data.

RESULTS

Participant characteristics

Table 1 indicates the main characteristics of the study participants. All children were Caucasian. Fourteen children with allergic rhinitis and asthma also had concurrent atopic dermatitis (21.5%). No statistically significant differences were found regarding demographic and anthropometric parameters. Lower spirometric values and higher FeNO levels were found in children with allergic rhinitis and asthma, the worst values in uncontrolled asthma (Bonferroni post-hoc testing, $p \leq 0.01$).

Principal component analysis of NMR spectroscopy datasets

The PCA was applied to urine and saliva NMR datasets. The first two components of both urine and saliva PCAs explained less than 35% of variation of the data (Figure 2). In order to evaluate the influence of allergic rhinitis and asthma as well as asthma control in the metabolic profile, the PCA score plot was colored according to these groups, but neither contributed to the main sources of variation of the data in both urine and saliva models (Figure 2).

Other potential factors that could have influenced the metabolic profile such as gender, age and body mass index were also investigated. None of them had any influence on the metabolic profile of either urine or saliva samples (Supplementary Figures S1, S2 and S3).

Univariable analysis followed by linear modelling

After Mann-Whitney U test, several variables were significantly different between healthy children and those with allergic rhinitis and asthma (Table 2). Subset selection by linear modelling reduced the number of variables; the best models chosen by logistic regression contained from five to eight variables (Table 2). Some metabolites contributing to these variables were identified (Table 3): arginine, taurine, citrate and aspartate (in saliva), and quinolinate, butyrate, pantothenate, gluconate, pseudouridine and lysine (in urine). Figures 3 and 4 show these differentiating variables levels in urine and saliva, respectively.

Associations between asthma differentiating spectral variables and clinical parameters

Variables including quinolinate, butyrate, pantothenate, and an unidentified metabolite correlated with spirometric parameters, while FeNO levels correlated with the variables comprised by quinolinate, gluconate and pseudouridine (Table 4). No correlations were found between salivary variables and spirometric parameters or FeNO.

Urinary quinolinate levels were higher in children with FEV_1 z-score<-1.64 ($p=0.015$), positive bronchodilation test ($p=0.002$) and multiple allergenic sensitization ($p=0.037$). Multiple sensitization was also associated with higher levels of the salivary variable comprising citrate and aspartate ($p=0.036$).

Lower levels of the salivary variable comprising arginine and taurine were found in children treated with montelukast on the previous week ($p=0.008$). No other associations were found between any of the differentiating spectral areas and drugs intake on the previous week, namely inhaled or nasal corticosteroids, inhaled bronchodilators or montelukast. No association was found between any differentiating spectral area and self-reported specific food intake or fasting time.

DISCUSSION

The exploratory metabolomics of urine and saliva resulted in differentiating subsets of NMR spectral features in children with allergic rhinitis and asthma, compared to healthy children. Moreover, some of these metabolic spectral areas were associated with lung function parameters, FeNO levels and multiple allergenic sensitization. Several metabolites contributing to some of these features were identified, stimulating further studies to validate the results and generating several hypotheses regarding their role in allergic rhinitis and asthma multimorbidity-related metabolic pathways to be further explored.

At variance with most of the previous metabolic profiling studies, we used untargeted metabolomics to uncover “unbiased” differentiating metabolic features of a specific asthma phenotype and considered allergic rhinitis and asthma multimorbidity in children for the first time. Furthermore, we have used two non-invasive samples, collected consecutively from each child, aiming for a more “systemic view”, yet totally non-invasive. A balanced sample of patients comprising different asthma control levels was studied, together with age and gender-matched non-atopic healthy children. All diagnoses were validated by specialist medical doctors and all children were characterized regarding airway inflammation measured by FeNO and lung function with spirometry and bronchodilation test. Spectral NMR areas of urine and saliva were associated with allergic rhinitis and asthma multimorbidity using statistical analyses that involved a pre-selection of variables followed by linear modelling. Compound identification was performed to benefit possible methodology validation, as well as raising pathophysiological hypotheses.

Molecular profiling by untargeted approaches may support the characterization of asthma phenotypes at the molecular level and contribute with new insights to the progress from clinical phenotypes to endotypes. Although diversity exists within the childhood “allergic asthma” phenotype[39], focusing on a defined clinical phenotype reduces clinical heterogeneity and may be useful in the molecular characterization of asthma “subtypes”, allowing easier comparison of results by different research groups. In fact, one probable justification for the non-replicated results obtained by the existing metabolic profiling studies in asthma may rely on the distinct study populations, especially considering the heterogeneity of asthma[4, 40].

Multivariable analysis by PCA of NMR spectra of urine and saliva showed no samples clustering according to allergic rhinitis and asthma multimorbidity, or asthma control. Similar results have been previously described in asthma profiling studies by other research groups using different analytical methods and biospecimens including urine, saliva, plasma and exhaled breath condensate (EBC)[6, 7, 9, 10, 14, 15, 41, 42], and may be due to the fact that the metabolome is sensitive to both internal and external influences that may be unrelated to disease status. It could also reflect heterogeneity in allergic rhinitis and asthma-related metabolic pathways or even still limited analytical coverage or discriminatory capacity to identify global shifts in multiple correlated metabolites. Yet, this does not mean that there are no metabolic variables that are useful to differentiate asthma. Feature selection can allow for the extraction of relevant metabolites[43]. This is applied to: a) reduce overfitting, b) improve model performance and c) gain a better understanding of the relationship between the metabolic features and the response clinical variable. Several strategies have been used by different research groups to identify metabolic discriminatory features classifying asthmatics from healthy controls. We chose univariable spectral area selection to measure the importance of each variable individually on allergic rhinitis and asthma in a simple and easy to interpret approach[41, 42, 44, 45]. These preselected sets of variables were further reduced by linear modelling and logistic regression was applied to select the final best set of spectral areas. As response variable, comparisons were made between healthy children and children with partly controlled and uncontrolled asthma, since more significant differences were expected to be found than in controlled asthma. Since partly controlled and uncontrolled asthma may be metabolically heterogeneous themselves, separate models were built for each group. In fact, by using this approach, distinct spectral areas and distinct metabolites

were identified that differentiated the two groups, except for pantothenate which was identified in urine as a relevant differentiating metabolite in both models. Different from previous metabolic profiling studies in asthma, we analyzed saliva and urine simultaneously from the same subject, but found distinct differentiating metabolites subsets in the two samples. However, some of the identified metabolites were indeed common to previous metabolomics profiling studies results and have been linked to asthma. In particular, arginine and fatty acid metabolism were previously reported pathways in asthma metabolomics studies, regarding both experimental animal models of allergic airway inflammation[46-49], and clinical studies in humans[7, 19, 21, 22, 24, 25].

An enrichment of pathways reflecting increased metabolism of amino acids in asthma has been anticipated[16]. Amino acids are mediators of immunological activities in asthma and may have antioxidant functions, namely taurine[20, 24]. Urinary citrate has been previously reported to reduce during asthma exacerbations[11]. Low arginine systemic levels have been described in asthmatics, which may be associated with induction of arginase activity by type 2 cytokines[21, 50]. Arginine also acts as a substrate for nitric oxide production[51], but we found no association between salivary arginine and FeNO levels. Although we found that these amino acids distinguished allergic rhinitis and asthma cases, the link with the reduced level of free metabolites in the saliva and these diseases is unknown. This could be related to systemic levels but also to dietary changes or salivary gland secretion. Of relevance, taurine is the most abundant free amino acid, mainly in proinflammatory cells and tissues exposed to elevated levels of oxidants, such as the salivary gland, where it exerts a role in regulation of salivary flow. Altered salivary flow rates have been described in asthmatics, which can contribute to changes in saliva composition[52, 53].

Urinary quinolinate is an endogenous metabolite of tryptophan at the kynurenine pathway, which has been linked to inflammatory pathways[54, 55]. Its levels were found to be higher in serum and lower in EBC in adult patients with allergic asthma[56]. Moreover, systemic quinolinate has been associated with eosinophilic cation protein, eosinophils in bronchoalveolar lavage fluid and peak asthma symptom scores after rhinovirus challenge[56]. Interestingly, besides a negative correlation with lung function parameters, we also found a positive association between quinolinate levels and multiple allergenic sensitization and FeNO levels, which could further support quinolinate as a marker in the type 2 inflammatory pathway[56]. However, the pathophysiologic mechanisms of enhanced systemic quinolinate in asthma remain to be determined. Urinary tryptophan has been also identified as a discriminatory metabolite in asthmatic children[7]. The immunomodulatory function of indoleamine 2,3-dioxygenase, the rate-limiting enzyme in tryptophan catabolism, and other compounds generated during tryptophan metabolism have also been described in asthma, supporting that further insights on this metabolic pathway may contribute to increased knowledge on asthma[20, 54, 56-58].

Butyrate has also been identified in exploratory EBC metabolomics in asthma[22, 25]. This molecule is a short chain fatty acid, mostly produced in humans in the gut by anaerobic fermentation of undigested carbohydrates and fiber polysaccharides. Besides the role in the energy requirement, butyrate is suggested to be a mediator in the communication between commensal microbiota and the immune system, with multiple anti-inflammatory effects, including suppression of nuclear factor kappa B activation and interferon gamma production, upregulation of peroxisome proliferator-activated receptor gamma and promotion of peripheral regulatory T cell generation, affecting effector cells migration, adhesion, proliferation and apoptosis[59-63]. It has been recently reported that butyrate may influence innate lymphoid cells proliferation and function, associated with reduced airway hyperactivity and inflammation[64]. The principle mechanism of action involves epigenetic regulation through the inhibition of histone deacetylase[59]. Overall, histone deacetylase inhibitors have been considered in the treatment of asthma and other inflammatory lung diseases[65]. The lower levels of butyrate were associated with partly and uncontrolled asthma and impaired lung function. Taken together, these findings may bring additional evidence to the role of gut microbiome in allergic asthma[66, 67].

In our study, using the identified differentiating spectral region subsets, the obtained summary scores showed good classification accuracies. While metabolites associated with allergic rhinitis and asthma in our study are biologically plausible and supported by previous experimental data, these preliminary

results require validation. This step is critical to assess true discriminatory ability and support for accuracy of the reported biomarkers in independent cohorts. Furthermore, essential steps in metabolomics studies design relate to monitoring external factors that may affect the metabolome and introduce significant bias to the results. External factors known to affect the metabolome include age, gender, body mass index and the circadian rhythm. Despite being well matched for age, gender, body mass index, and all samples being collected at the same time of the day, it is important to highlight that diet and current asthma treatment may have interfered with the urine and saliva metabolic profile in our study. Although care has been taken to refrain from drinking or food intake before samples collections and most medication was inhaled and had been stopped for at least 12 hours, we cannot firmly rule out that our results were not affected by diet or medication. Nevertheless, no association was found between any differentiating spectral area and self-reported specific food intake or fasting time or drug use on the previous week, except for oral montelukast which was associated to the variable comprising arginine and taurine in saliva. However, an explanation for this association is unknown to us, considering that montelukast had been taken more than 36 hours before saliva collection. Randomized trials and animal model studies analyzing the effect of medication on the metabolic profile can help distinguishing the effect of drugs from the disease effect on the metabolome[17].

In summary, this exploratory study supports the potential of untargeted metabolomics of non-invasive urine and saliva samples in the “unbiased” molecular characterization of childhood allergic rhinitis and asthma multimorbidity. We have identified a subset of the NMR spectral areas of urine and saliva significantly different in children with allergic rhinitis and asthma compared to healthy controls. Some of these metabolic variables were associated with such clinical readouts as lung function parameters, FeNO levels and multiple allergenic sensitization. Moreover, several metabolites contributing to the differentiating variables were identified, stimulating further studies to validate the results and generating hypotheses to be further explored, namely regarding the role of the previously reported asthma-related arginine, taurine, quinolinate and butyrate metabolites in allergic rhinitis and asthma multimorbidity.

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Table 1: Participants characteristics

CHARACTERISTICS	Healthy children (n=24)		Children with allergic rhinitis and asthma multimorbidity						p-value
			Controlled asthma (n=20)		Partly controlled asthma (n=21)		Uncontrolled asthma (n=24)		
Age months, median (min-max)	116.0	(73.0-155.0)	105.0	(74.0-155.0)	115.0	(76.0-148.0)	128.0	(78.0-155.0)	0.106
Gender male, n (%)	12	(50.0)	10	(50.0)	15	(71.4)	13	(54.2)	0.439
BMI Kg/m^2, median (min-max)	16.87	(12.85-22.20)	16.65	(13.66-23.40)	17.71	(13.31-22.79)	18.52	(14.18-24.61)	0.092
Multiple sensitization, n (%)	0	(0)	11	(55.0)	13	(61.9)	15	(62.5)	0.860*
Nasal topic corticosteroid use, n (%)	0	(0)	6	(30.0)	8	(38.1)	9	(37.5)	0.832*
Inhaled corticosteroids use, n (%)	0	(0)	12	(60.0)	14	(66.7)	11	(45.8)	0.351*
Inhaled LABA use, n (%)	0	(0)	6	(30.0)	4	(19.0)	10	(41.7)	0.259*
Oral montelukast use, n (%)	0	(0)	0	(0)	3	(14.3)	4	(16.7)	0.201*
Syst. corticosteroid < 6 months, n (%)	0	(0)	0	(0)	4	(19.0)	4	(16.7)	0.128*
FEV_1/VC z score, mean (SD)	0.79	(0.63)	0.21	(1.32)	-0.83	(1.55)	-2.63	(2.12)	**<0.001**
FEV_1 z score, mean (SD)	0.85	(1.07)	1.26	(1.65)	0.19	(1.30)	-0.88	(1.42)	**<0.001**
MMEF z score, mean (SD)	-0.03	(0.90)	-0.38	(1.39)	-1.52	(1.50)	-2.88	(1.44)	**<0.001**
Positive bronchodilation test, n (%)	1	(4.2)	0	(0)	6	(28.6)	18	(75.0)	**<0.001**
FeNO, median (min-max)	5	(0-24)	10	(0-87)	15	(0-94)	29	(7-104)	**<0.001**

BMI – body mass index; FeNO – fractional exhaled nitric oxide; FEV_1 – forced expiratory volume in one second; LABA – long acting beta 2 agonist; min-max – minimum-maximum; MMEF – maximum midexpiratory flow; SD – standard deviation; Syst. – systemic; VC – vital capacity.

* - comparative analysis considering only participants with allergic rhinitis and asthma

Table 2: Variables preselection and characteristics of multivariable models for differentiating subsets of spectral variables classifying children with allergic rhinitis and asthma from healthy controls

Groups	Sample	Mann-Whitney U test	Logistic regression final model				
		Number of significant bins	Number of significant bins	LR chi^2	R^2	AIC	AUROC
Healthy vs Uncontrolled asthma	Urine	29	8	54.23	0.903	30.31	0.988
Healthy vs Partly controlled asthma	Urine	19	5	48.07	0.877	26.11	0.978
Healthy vs Uncontrolled asthma	Saliva	43	5	41.71	0.785	35.42	0.955
Healthy vs Partly controlled asthma	Saliva	15	6	31.14	0.677	43.77	0.921
AIC – akaike information criterion; AUROC – area under the receiver operating curve; LRch^2 – likelihood ratio qui-squared							

Table 3: Identified allergic rhinitis and asthma differentiating metabolic variables

Groups	Sample	Metabolites	Odds-Ratio (2.5%-97.5% confidence interval)
Healthy vs Uncontrolled asthma	Urine	Quinolinate	2.69 (1.10-8.90)
		Butyrate + Pantothenate	0.41 (0.16-0.87)
		Gluconate + Pseudouridine	2.30 (1.10-5.69)
Healthy vs Partly controlled asthma	Urine	Pantothenate	0.19 (0.05-0.48)
		Lysine	9.90 (1.80-79.90)
Healthy vs Uncontrolled asthma	Saliva	Citrate + Aspartate	0.41 (0.17-0.81)
Healthy vs Partly controlled asthma	Saliva	Arginine + Taurine	0.51 (0.23-0.98)

Table 4: Correlations between differentiating spectral variables and lung function parameters (n=89)

Spectral features (metabolites)*	Spearman rho (p)											
	FeNO (ppm)		FEV_1/VC (z-score)		FEV_1 (z-score)		MMEF (z-score)		Bronchodilator change in FEV_1 (L)		Bronchodilator change in FEV_1 (%)	
Quinolinate	**0.240**	**(0.024)**	**-0.314**	**(0.003)**	-0.157	(0.141)	**-0.282**	**(0.007)**	**0.306**	**(0.004)**	**0.366**	**(<0.001)**
Gluconate+Pseudouridine	**0.230**	**(0.030)**	-0.163	(0.128)	-0.056	(0.604)	-0.133	(0.213)	0.163	(0.126)	0.179	(0.093)
Butyrate + Pantothenate	-0.178	(0.227)	**0.277**	**(0.032)**	0.142	(0.183)	0.209	(0.050)	**-0.263**	**(0.013)**	**-0.227**	**(0.032)**
X0.8402 (unknown)	-0.162	(0.128)	**0.227**	**(0.033)**	0.040	(0.711)	**0.222**	**(0.036)**	**-0.228**	**(0.032)**	**-0.216**	**(0.042)**

FeNO – fractional exhaled nitric oxide; FEV_1 – forced expiratory volume in one second; MMEF – maximum midexpiratory flow; VC – vital capacity

* - only spectral features with significant correlations are shown

Figures with legends:

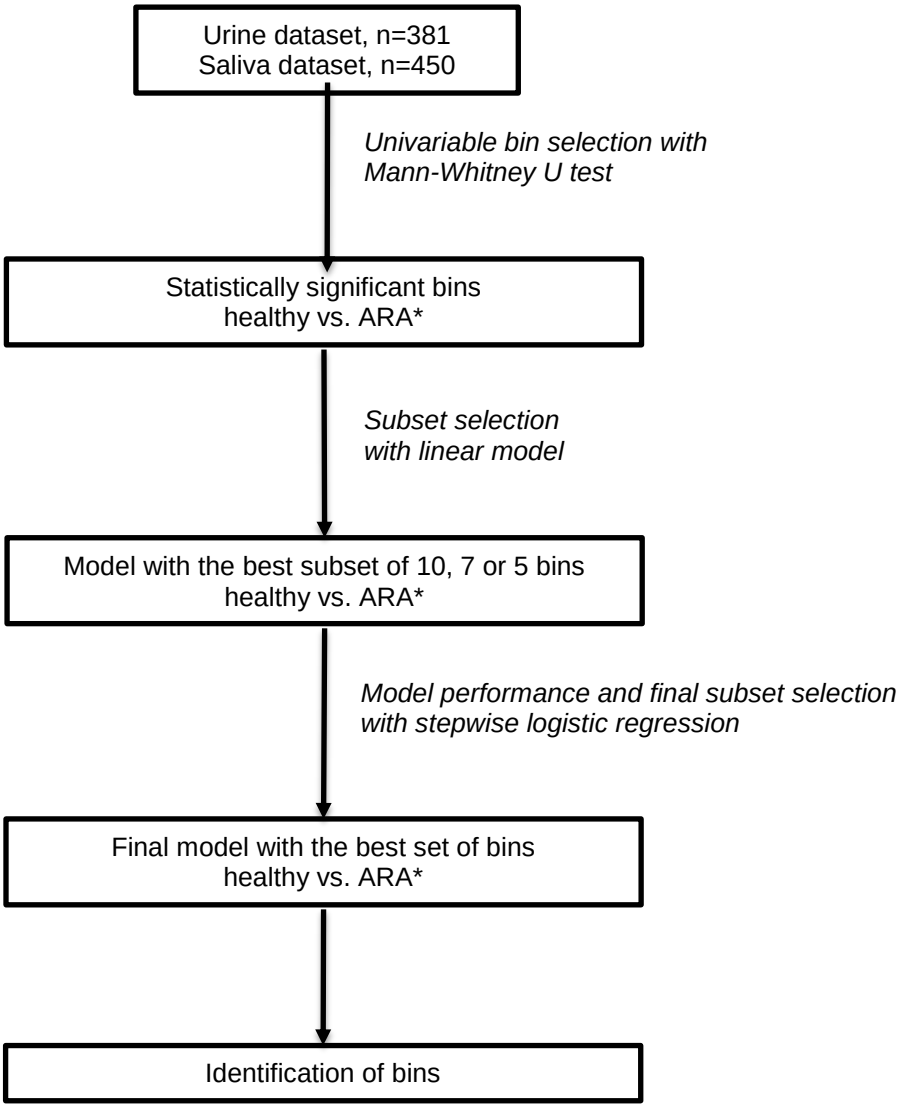


Figure 1 – Data analysis workflow (* ARA: allergic rhinitis and asthma, considering uncontrolled asthma or partly controlled asthma).

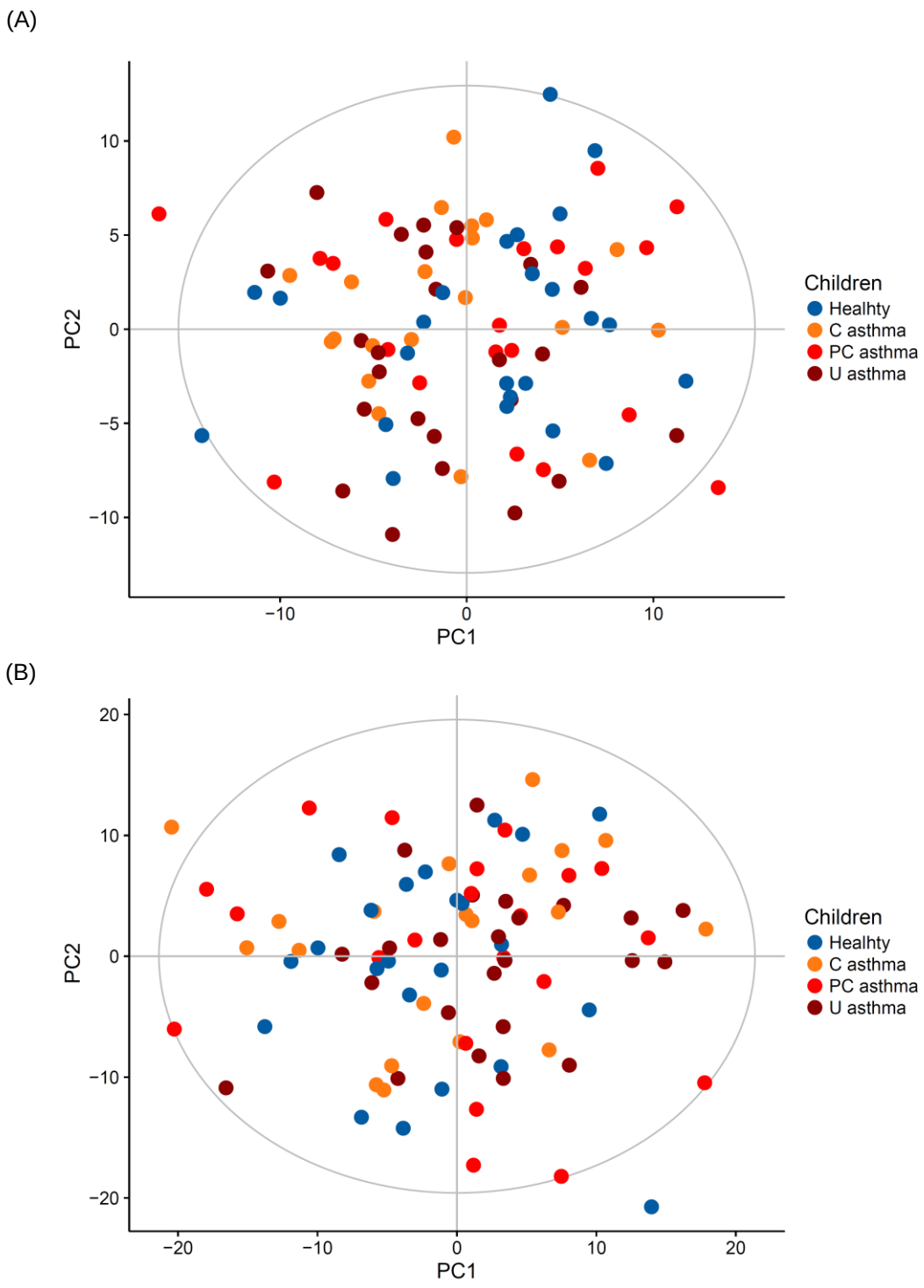


Figure 2 – Principal component analysis score plots of urine (A) and saliva (B) samples. Samples are colored according to disease status and asthma control. The first two components of the models explained 17% and 33% of the variation of the urine and saliva data, respectively.

C: controlled; PC: partly controlled; U: uncontrolled.

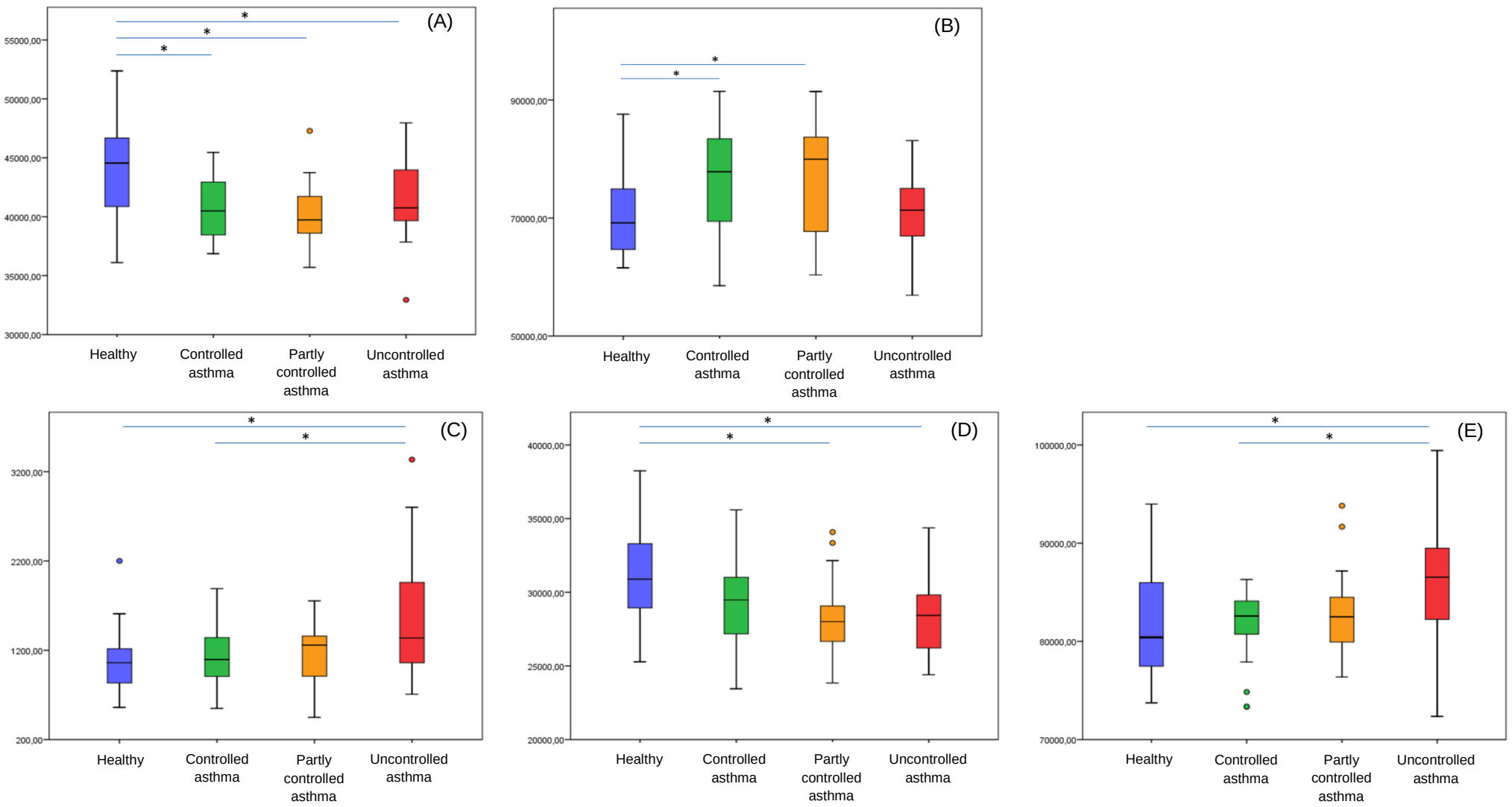


Figure 3 – Boxplots of identified metabolites in urine to discriminate healthy children from children with allergic rhinitis and asthma, stratified according to asthma control: (A) pantothenate; (B) lysine; (C) quinolinate; (D) butyrate + pantothenate; (E) gluconate + pseudouridine; (n=89, * p<0.027).

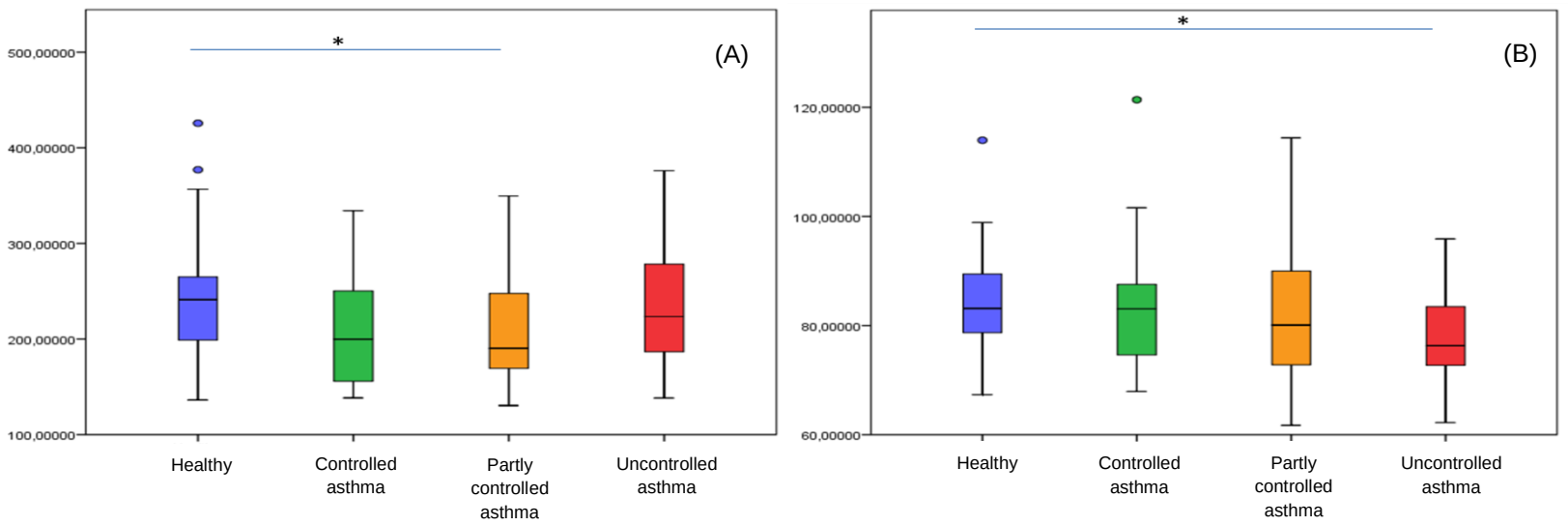
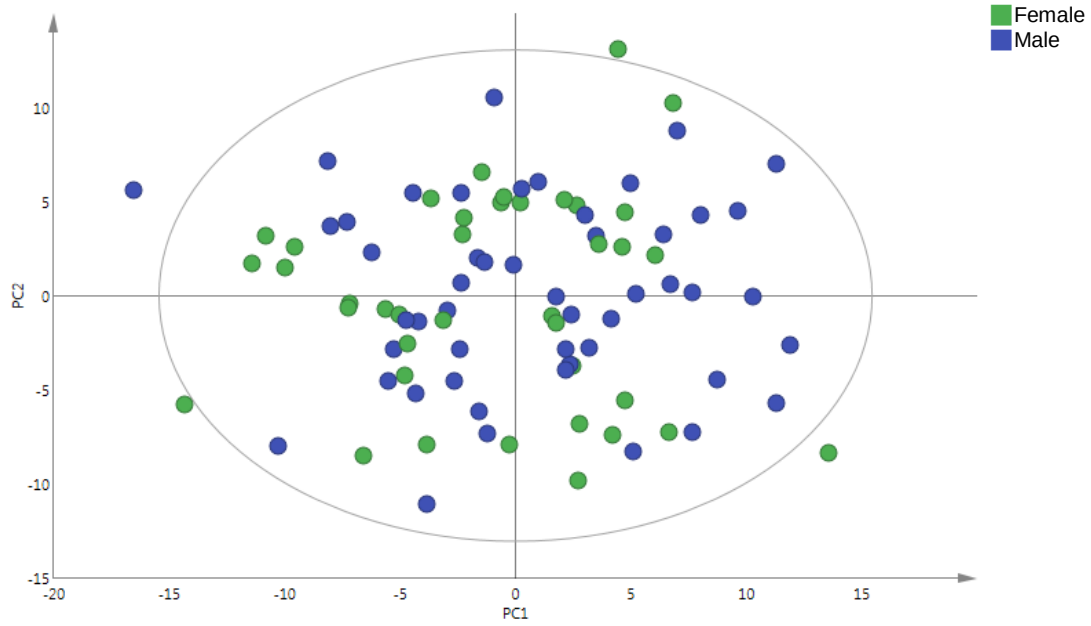


Figure 4 – Boxplots of identified metabolites in saliva to discriminate healthy children from children with allergic rhinitis and asthma, stratified according to asthma control: (A) arginine + taurine; (B) citrate + aspartate; (n=88, * p<0.042).

Supplementary file

(A)



(B)

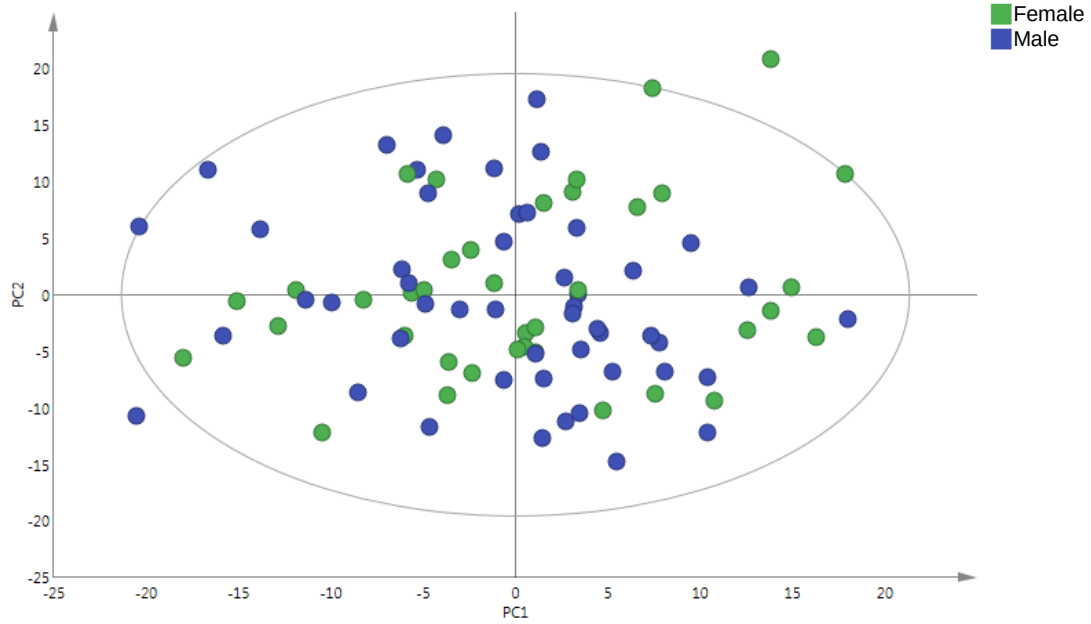
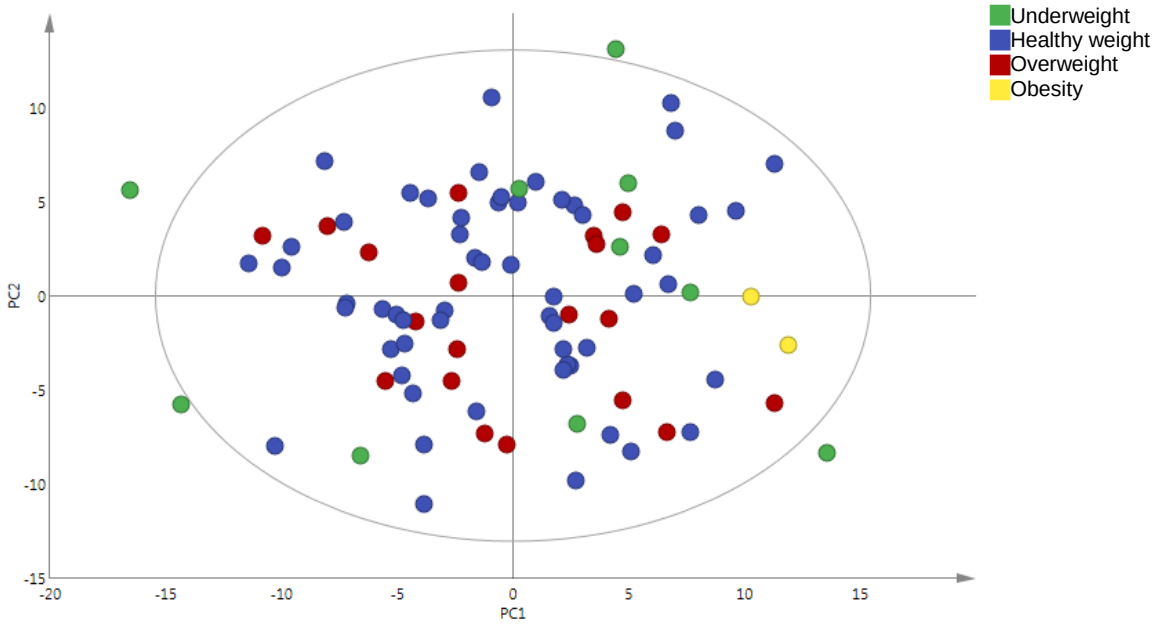


Figure S1 – Principal component analysis score plot of urine (A) and saliva (B) samples, colored according to gender (Legend: 0 – female; 1 – male).

(A)



(B)

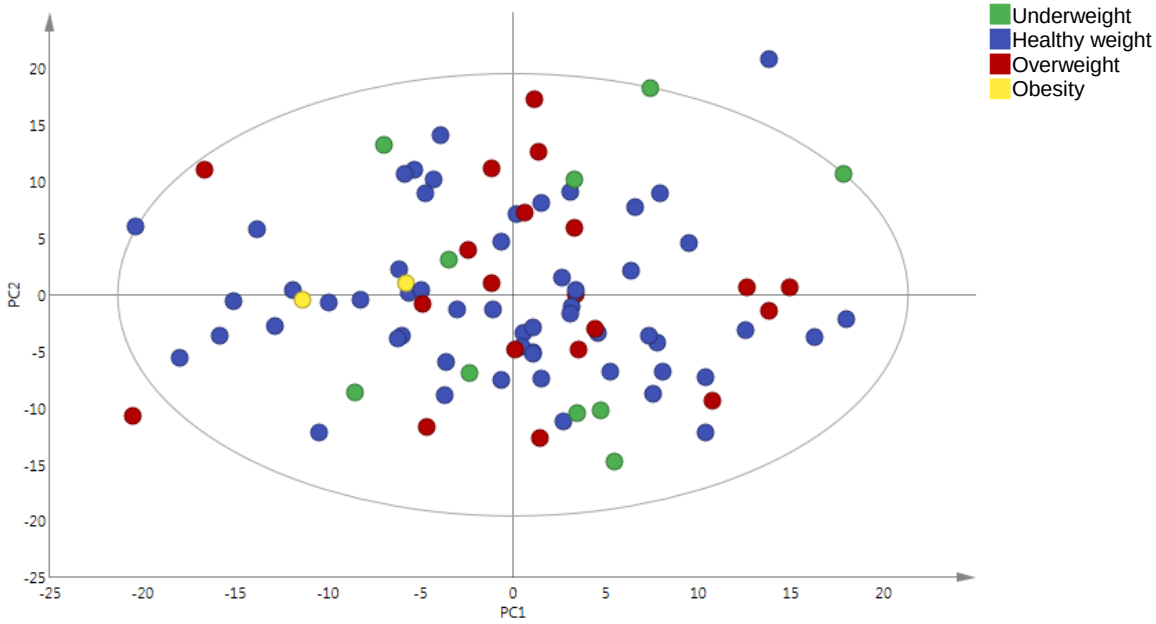
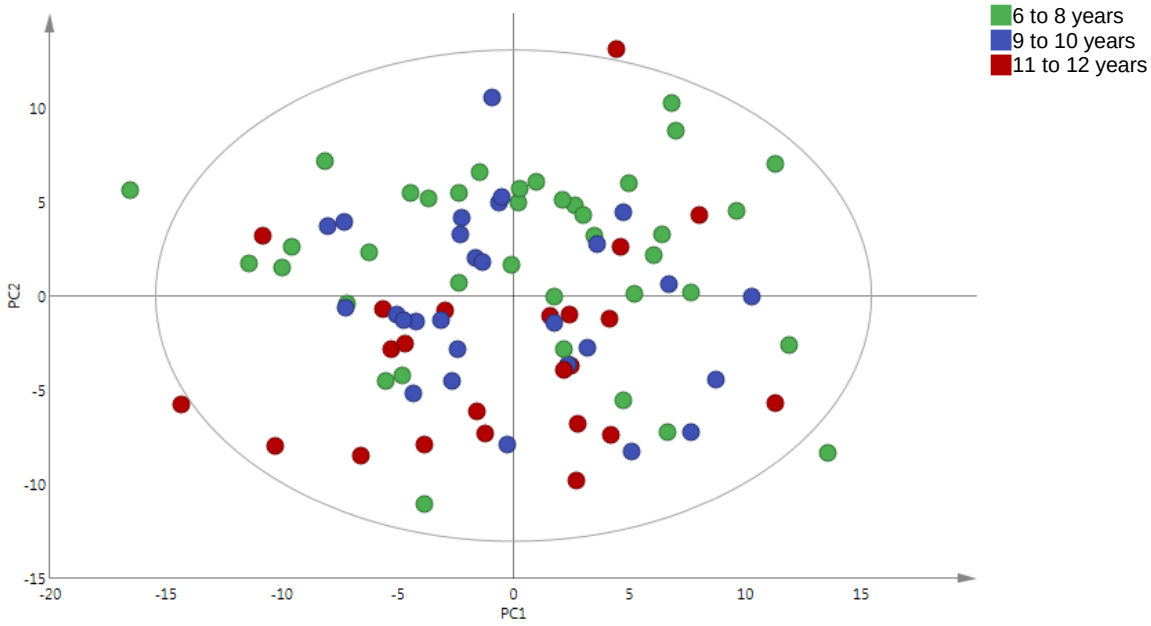


Figure S2 – Principal component analysis score plot of urine (A) and saliva (B) samples, colored according to body mass index. (n.b. Partial least-squares regression models were tried without success.)

(A)



(B)

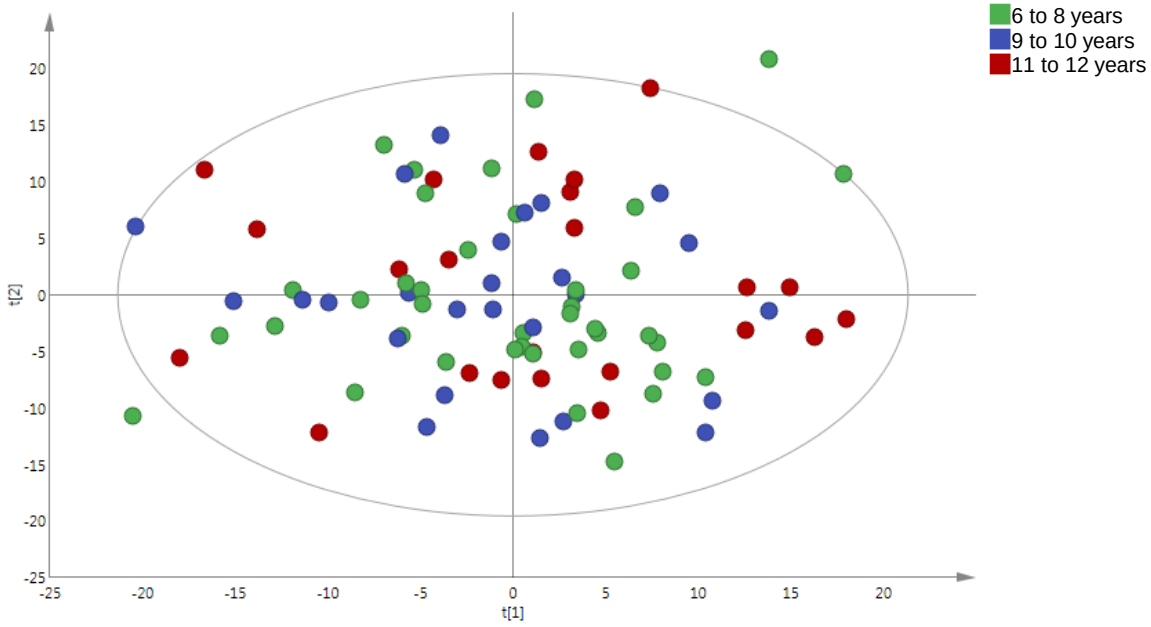


Figure S3 – Principal component analysis score plot of urine (A) and saliva (B) samples, colored according to age groups. (n.b. Partial least-squares regression models were tried without success.)

Output 7:

Room air controls in exhaled breath condensate metabolic profiling

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The recently updated European Respiratory Society technical standards on exhaled biomarkers provide methodological recommendations of utmost importance for comparable results and potential use of exhaled biomarkers in clinical practice and research[1]. Exhaled breath condensate (EBC) composition can be influenced by several factors that arise during sample collection, storage and processing. One of these factors is the inhaled air composition[1]. Yet, ambient air controls have not been routinely reported in EBC studies. Addressing potential exogenous compounds in EBC becomes even more important with the growing number of "omics" studies using untargeted approaches with such multivariable analytical techniques as nuclear magnetic resonance (NMR) and mass spectrometry. In a seminal paper on NMR profiling of EBC, Bertini et al used a homemade EBC collecting device[2]. Humidified room air was pulled into the condensing apparatus and the analysis of this sample resulted in NMR spectra free of ambient air or device contamination signals[2]. On the other hand, using the standard commercial device EcoScreen®, Izquierdo-García et al have reported the presence of a number of exogenous compounds in the room air samples, including, among other components, acetate, formate, propionate, lactate, benzoate, glycerol and propylene glycol[3]. Moreover, ambient air NMR profiles from the same collection room varied depending on the time of day or day of collection[3].

In our study, we used the disposable RTube™ collection system (Respiratory Research, Inc.; Virginia, USA), cooled to -20ºC. EBC samples were collected on two different days from seven volunteers (two children and five adults; three with asthma). During collection, subjects breathed at normal frequency and tidal volume for 10 minutes, wearing a nose clip and were asked periodically to swallow saliva. In total, four room air samples were collected simultaneously with the EBC samples in two ways: (a) two samples using a calibrated syringe connected to the RTube mouthpiece, pumping 500ml of room air into the collection system at a flow rate of approximately 8L/min for 10 minutes, reproducing normal human breathing; (b) two samples using a vacuum pump connected to the outlet of the collection system for 10 minutes, thus avoiding possible contaminations from the syringe material. All sampling procedures were carried out during the morning, in the same room (volume: 36m^3; with air conditioning set to 24ºC). Collected samples were immediately stored in sterile high-grade polypropylene cryovials at -80ºC until their analysis by NMR. On the day of analysis, samples were thawed at room temperature and insoluble material was spun down by centrifugation. The supernatants were mixed with phosphate buffer (pH = 7.4) in deuterated water, in a 9:1 ratio of sample/buffer, and ^{1}H NMR spectra were recorded in a 14.1 T NMR spectrometer (Bruker, Avance II); the NMR method was optimized according to Bertini et al.[2]. Figure 1 shows the representative ^{1}H-NMR spectra of the room air samples collected using the vacuum pump or the syringe (both spectra were identical) and the EBC sample. The composition of the room air spectrum was similar to the one from EBC, indicating a possible contamination from each sample to the other. Several reasons could be considered to explain these results, from environmental contamination to the effects of the condensing equipment and samples storage or processing methodology. Although the influence of each of these factors has not been analyzed individually, our spectra were similar to Bertini et al published EBC spectra, using similar analytic procedures[2]. Furthermore, the RTube® collection system is made of inert materials and the NMR spectra of water from this collecting system has been shown to be clearly different from EBC spectra[4]. These strengthened a possible contamination of room air and EBC samples in our study. A number of possible factors could have contributed to the potential environmental contamination, including the limited space in the collection room, insufficient room ventilation and the exhaled breath of the assisting personnel. Subtracting the air background from the breath signal wasn't feasible. This method is increasingly used in mass spectrometry breath analysis by measuring the relative abundance of the metabolites in exhaled breath and in ambient air, but such approach may not be easily translated to NMR-based metabolomics data. A solution implemented in the previous reports was the use of suitable filters[1]. Izquierdo-García et al placed a trap for air contained water-soluble organic compounds in the EBC collecting system, which removed most of the NMR spectral air signals[3]. A similar strategy has been used by Motta and colleagues, who coupled a respiratory protection particulate

filter to EcoScreen® and TURBO-DECCS® condensers and obtained no NMR signals in the condensed room air[5,6]. Thus, pumping filtered air into the collector using high efficiency filters (against particles with approximately 0.3µm mass median aerodynamic diameter) gave clean reference spectra, independent of the sampling device. However, this provides no insight into the composition of the air inhaled by a patient. Another possibility would be the use of a standard air source during EBC collection. Although variable interaction between this source of air and EBC is inevitable, at least this procedure would better allow independence from room air variability.

Our results reinforce the need to address potential exogenous components in EBC profiling studies. This can be particularly difficult in NMR-based metabolomics studies. Inhaled air sample controls collected simultaneously with EBC samples are essential as well as methodologic procedures that allow distinguishing exogenously originated metabolites in this matrix.

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Figure 1:

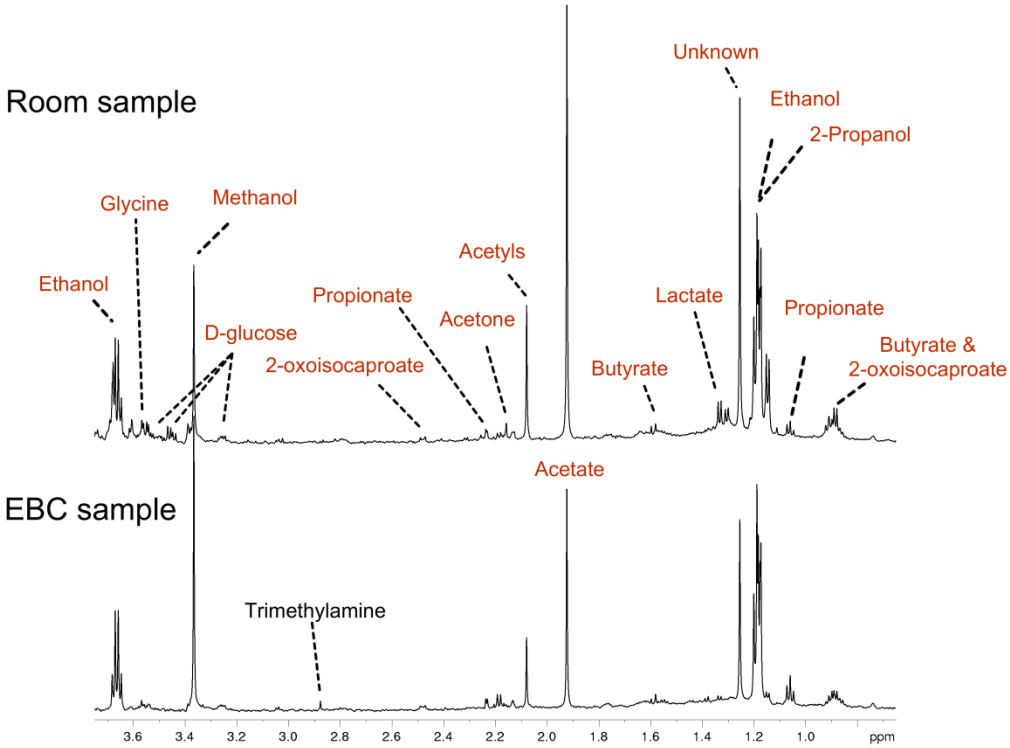

Figure 1 – Region of ^{1}H 1D nuclear magnetic resonance spectra from ambient room air (up) and exhaled breath condensate (bottom). The metabolites annotated in red were found in both samples.

Chapter 5: Discussion

A high prevalence of asthma was found in children (prevalence of current asthma 8.4%) and in the elderly (prevalence of physician-diagnosed asthma 10.9%) in the Portuguese population. For the first time, nationwide prevalence of rhinitis was estimated in the elderly and a high frequency was found (29.8%). Strong associations between rhinitis and asthma were described at the population-level in these two age groups. Despite being common diseases, these studies suggested that rhinitis and asthma care had been substandard, highlighting the need for a clinical integrated, global assessment of asthma and rhinitis multimorbidity also in children and the elderly.

Atopy and rhinitis at preschool ages were independent risk factors for asthma persistence in adolescence, based on “unbiased”, multidimensional modelling of data from a cohort of children with preschool recurrent wheezing. Multimorbidity, particularly rhinitis with or without associated atopy, tended to predict a worse prognosis of symptoms persistence and impaired lung function in later childhood and adolescence, suggesting the demand for distinct management approaches. These studies further suggested the need for an integrated care pathway of asthma and rhinitis together, since the very beginning of symptoms.

The consistent and independent correlation found between PNIF and lower airway patency (PEF and FEV_1) in school-aged children may reflect the physiologic background for the common rhinitis and asthma multimorbidity. These independent correlations also suggested that, ideally, at least PEF values should be considered, besides age and gender, when evaluating nasal obstruction by means of PNIF in this age group. The objective evaluation of nasal function by PNIF provided complementary objective information to subjective control assessment in children with allergic rhinitis and asthma, in an integrated care pathway approach.

Ultimately, the application of exploratory metabolomics approaches to uncover “unbiased” differentiating metabolic features in non-invasively collected samples of urine and saliva in children with allergic rhinitis and asthma was demonstrated to be feasible. Differentiating NMR spectral areas were suggested to be associated with the allergic respiratory multimorbidity in children. Some of these metabolic variables were associated with lung function parameters, FeNO levels and multiple allergenic sensitization. Moreover, several metabolites contributing to the differentiating variables were identified, stimulating further studies to validate the results and generating hypotheses to be further explored, namely regarding the role of the previously reported asthma-related metabolites arginine, taurine, quinolinate and butyrate in allergic rhinitis and asthma multimorbidity. The EBC metabolic profile was found to be highly comparable to ambient air spectral composition, reinforcing the importance of room ambient air control during samples collection and the need for analytical procedures to differentiate exogenously originated metabolites in EBC.

The estimated prevalence of current asthma in children was 8.4% (95%CI 6.6%-10.7%), using the pediatric-specific data from the population-based, all-age, nationwide telephone interview INAsma study. The prevalence of rhinitis and of physician-diagnosed asthma were estimated to be 29.8% (95%CI 28.4%-31.3%) and 10.9% (95%CI 9.9%-11.9%), respectively, in the epidemiologic, nationwide, face-to-face interview elderly-targeted study. A strong association between asthma and rhinitis was found both in children and in the elderly.

These studies were the first population-based epidemiologic studies aimed to estimate the asthma and asthma-like symptoms prevalence in Portugal among all pediatric ages (<18 years) and to estimate the prevalence of rhinitis and physician-diagnosed asthma in the elderly (>65 years), respectively. Representative samples of the Portuguese population were included. These two extremes of life were particularly vulnerable, internationally data lacking groups. Few nationwide studies on the prevalence of asthma across the entire pediatric population had been performed[55, 56], and no previous study had reported the prevalence of asthma-like symptoms across all pediatric ages. On the other hand, nationwide epidemiologic data on rhinitis in the elderly did not exist at the international level. Our study was the first epidemiologic study exclusively dedicated to the elderly that concurrently evaluated rhinitis and asthma symptoms, reporting an association according to ARIA classification of rhinitis severity and duration.

Both studies contributed to assess nationwide diseases prevalence in these population groups. However, several limitations need to be considered:

a) Primary endpoint: In the pediatric study, the survey was not designed for children but rather for the overall Portuguese population. Although the questions included in the survey and the definitions used were similar to other previous pediatric studies[130, 131], the sample size was calculated for the total Portuguese population and not for the pediatric population. In this study, the inclusion of 716 children allowed to estimate current asthma prevalence with an error of 2% (precision) in 95% confidence interval.
   The elderly-targeted study was designed to estimate rhinitis prevalence. The frequency of physician-diagnosed asthma was a secondary endpoint and data on lower airway symptoms was not collected. Yet, the inclusion of 3678 subjects allowed the estimation of physician-diagnosed asthma prevalence with an error of 0.9% (precision) in 95% confidence interval.
b) Sampling methodology: In both studies, the use of the 2001 National Census for sampling could not account for the reduction in the number of births or the increase in the elderly population that occurred in the next decade; however, it was the most recently available data at the time of data collection. Nevertheless, the sampling strategies allowed that the number of each gender, age and Portuguese region were reliably correspondent to the structure of the resident population in Portugal[132].
c) Diagnoses confirmation: Both studies were limited by the absence of physician evaluation and of objective biomarkers of lung function and atopy. Therefore, participants' misclassification can be expected, as in any similar epidemiologic surveys and classification of allergic/non-allergic diseases is impaired.
d) Questionnaire application: Other factors, such as recall or information biases may have also influenced the results. Nevertheless, the use of short and simple questionnaires, applied by trained interviewers, facilitated the inclusion of participants. It also minimized difficulties in reading or comprehending and the loss of compliance throughout the interview to reduce missing data. Furthermore, in the pediatric study, answers were given by the main caregiver for participants

aged <15 years and by the adolescent when the participant was ≥15 years. This may limit comparisons between these age groups. Changes of parent-reported to auto-reported outcomes are debatable but recommended between school-age and adolescence[133].

Epidemiologic studies at the population level are important to increase knowledge on a disease or condition and to better define adequate health policies. However, prevalence estimates are highly dependent on the used definitions of a disease. Standardized operational definitions of asthma and rhinitis are lacking, which may lead to different estimates and preclude study results comparisons. In our study in children, a GA^{2}LEN-adapted questionnaire was used, including the ECHRS questions on asthma symptoms[46, 49]. Current asthma was defined as lifetime asthma (i.e., a positive answer to "*Has the child ever had asthma?*") plus at least one of three associated symptoms or medication use: "*wheezing*", "*waking with breathlessness*" or "*having an asthma attack*" in the previous 12 months, or "*currently taking asthma medication*". However, asthma prevalence varied according to different used definitions, from 6.3% (lifetime asthma plus wheezing in the last 12 months) to 9.1% (lifetime asthma plus any asthma-like symptom in the last 12 months or current asthma medication). This highlights the impact of the asthma definitions used on its prevalence estimates[134, 135]. Wheezing is many times used as a proxy of asthma but it can be caused by many conditions other than asthma. Using wheezing alone as a proxy of asthma diagnosis can overestimate asthma prevalence, particularly in children aged <3 years[136]. Wheezing-based asthma definitions are also limited by differences in the concept and perception of wheezing, with different meanings among parents and health professionals[137]. A more global description of asthma symptoms may be necessary to reduce the risk of misclassification of asthma. Thus, we have used composite measures and reported the different operational estimates, to allow for more accurate comparisons between studies. Still, standardization of epidemiologic asthma definitions and their clinical validation are needed[135].

Our estimate of lifetime asthma in children was 11.2% (95%CI 9.1%-13.7%). It was based on the same question of the ISAAC study. The estimate in school-aged children was higher than that in the 6 to 7-years-old age group in the Portuguese ISAAC survey (12.3% versus 9.8%), while our estimate for the adolescents was similar to that in the 13 to 14-years-old age group in the ISAAC survey (13.9% versus 13.8%)[50]. In a cross-sectional, questionnaire-based (ISAAC-adapted) large survey including 5003 children aged between 3 to 5 years living in mainland Portugal (ARPAkids study, performed in 2007), the prevalence of physician diagnosed asthma was 4.6% (95% CI 4.0%-5.2%)[61]. Another study performed in 2010 reported similar estimates of physician diagnosed asthma (4.6%, 95%CI 3.9%-5.4%) in 3186 preschool children attending day care centers in Lisbon and Porto[138]. Although the operational definitions of asthma were not the same, these estimates are not much distant from our results reporting current asthma in preschool children (6.5%, 95%CI 3.5%-11.6%).

The prevalence of asthma has been shown to vary in the different countries. The prevalence of doctor diagnosis of lifetime asthma in the German pediatric population was 4.7% (using data from 2003-2006)[55]. This prevalence is much lower than ours but it is in agreement with the estimated prevalence of lifetime asthma among German children reported in the ISAAC study (4.3% in 6-7 years-old and 7.5% in 13-14 years-old children in Germany, versus 9.8% and 13.8% in Portugal, respectively)[139]. Data from the 2010 National Health Survey for children aged <18 years in the United States of America reported an estimated doctor diagnosed lifetime asthma prevalence of 13.6% and current asthma of 9.5%[56], which are similar to our estimations.

In the elderly-targeted study, we could not present the more accurate composite measures of asthma. The lack of information regarding lower airways symptoms precluded these definitions. Therefore, current asthma prevalence, as defined in the INAsma study, could not be estimated. Physician-diagnosed

asthma on current treatment (estimated prevalence 7.6%, 95%CI 6.7%-8.4%) may be a proxy of the current asthma definition used in the pediatric study and thus suggest similar frequencies of the disease in the pediatric and in the elderly Portuguese population. Furthermore, the estimated prevalence of physician-diagnosed asthma on current treatment did not differ significantly from the estimated prevalence of current asthma in the INAsma subgroup analysis of subjects aged >65 years (8.0%, 95%CI 6.7%-9.5%)[53], suggesting that this might be a reliable approximation of the real prevalence in the Portuguese population. However, such direct comparisons may not be accurate, given the different criteria used for the definitions of asthma. In the INAsma study, the subgroup analysis by age groups considering children (aged <18 years), adults (18-65 years) and the elderly (>65 years) showed no statistically significant difference in the prevalence of current asthma in Portugal[53].

Adjusting for possible confounding factors, the prevalence of asthma in the INAsma study was lower in elderly subjects who reported no heart disease[53]. Since heart disease is common in the elderly and may be a cause of respiratory symptoms, the authors pointed out the possibility of an overestimation of current asthma in elderly subjects with cardiac disease. Asthma prevalence estimates did not change after adjusting for chronic bronchitis[53]. In the present study, in spite of the limitations due to physician-diagnosed asthma being only self-reported and the lack of information on environmental exposures including tobacco smoke, the fact that a doctor's diagnosis and current asthma treatment were considered could have helped to reduce bias and confounding factors related to respiratory symptoms caused by other conditions, such as heart disease, bronchitis or chronic obstructive pulmonary disease (COPD). Nonetheless, distinguishing asthma from COPD can be particularly difficult. COPD is often assumed to be an "aging-related disease", leading physicians to diagnose COPD rather than asthma in the elderly[140-142]. Conversely, other studies report asthma overdiagnosis in adults, including COPD misdiagnosis with asthma[143-145]. Phenotypic overlap between these two main chronic obstructive diseases justified the recent adopted nomenclature of asthma-COPD overlap (ACO)[6, 146].

In multivariable analysis, asthma prevalence was similar in both genders and according to municipality typology, in accordance with the INAsma study[53]. Unlike suggested by other studies[141, 147], the prevalence of asthma in the elderly did not diminish with age. However, we cannot exclude that our results may be influenced by an increasing tendency for asthma misclassification with COPD or other mimicking conditions, with increasing age[143-145], which should be explored.

In other epidemiological studies targeting elderly subjects from other countries, the prevalence of asthma varied between 3.6% and 7.6%[147-157]. However, the different selection criteria and definitions of asthma limit inter-studies comparisons. Asthma overdiagnosis or the other stated biases may have concurred for the higher prevalence obtained in this study. The clinical validation of the epidemiologic definition of asthma in this age group is also needed. A study including medical evaluation, information on tobacco and other environmental exposures, and lung function parameters would be of utmost importance to address this issue in the elderly.

Regarding rhinitis, population-based studies in the elderly are very scarce. Only one study performed in Mexico City had previously shown that allergic rhinitis was present in 3.6% of 333 elderly subjects who were attending a social security recreation center[149]. In a more recent community-based elderly population cohort study in Korea including 982 elderly adults, the prevalence of current rhinitis was 25.6%[158]. In all-ages epidemiologic studies, subgroup analysis reported rhinitis prevalence between 13.0% and 25.9% in the elderly[86, 148, 159-161]. However, similarly to epidemiologic asthma studies, different selection criteria and rhinitis definitions restrict inter-study comparisons. Our present findings are in agreement with the previous study reporting rhinitis prevalence in adults in Portugal[86]. That study, performed in 2004, using questions and a rhinitis definition that were similar to those used in the present report, found high rhinitis prevalence in adults aged 16–95 years (26.1%). In the same study[86], a

subpopulation analysis found that rhinitis prevalence in subjects aged above 65 years was 25.9%. The higher prevalence observed in the present study (29.8%) may reflect either a more accurate estimation of rhinitis in the elderly or an actual increasing tendency in rhinitis prevalence in this age group over the years. In the INAsma study, 22.1% of included subjects had rhinitis (the same percentage as found in children)[53], but direct comparisons are limited because, again, different sampling and definitions for rhinitis were used. "Allergic rhinitis" was defined in the INAsma study as a positive answer to the question: *"Do you have any nasal allergies, including hay fever?"*. This is clearly different from the composite definition used in the study in the elderly group (i.e., presence, usually or in the last 12 months, of at least two of the following symptoms: "*repeated sneezing and itchy nose*", "*blocked nose for more than one whole hour*", or "*runny nose when not having a cold or flu*"), based on ECRHS and ARIA[23, 162]. This definition, requiring the presence of at least two nasal symptoms, can suggest the diagnosis of allergic rhinitis together with the fact that 68.6% of the elderly had concomitant complaints of red eye, eye pruritus and epiphora, suggesting allergic rhinoconjunctivitis. However, the definitions of allergic rhinitis and allergic rhinoconjunctivitis were not used because they require the assessment of the specific immunological mechanisms involved and that was not part of this study. Other rhinitis subtypes could not be excluded. Still, this definition of rhinitis was important to rule out some of other causes of nasal symptoms, namely the so-called rhinitis of the elderly or senile rhinitis when characterized by clear rhinorrhea without other nasal symptoms[23, 26]. We also found a higher prevalence of rhinoconjunctivitis than that previously reported in adults (18.4%)[86]. A study comparing the characteristics of allergic rhinitis in younger and older patients suggested that elderly patients have rhinitis plus conjunctivitis more frequently than young adults[163].

The link between asthma and rhinitis was relevant in the children and in the elderly. Our data contributed to support this association at the population level in pediatric and older ages. The presence of rhinitis in children was associated with a higher prevalence of having any asthma-like symptom (OR 3.67, 95%CI 2.52-5.39). A strong association was found between current asthma and rhinitis in children (OR 5.20, 95%CI 3.05-8.85). On the other hand, about 80% of the elderly subjects diagnosed with asthma had rhinitis; among elderly subjects with rhinitis, 30% reported asthma diagnosed by a doctor. The prevalence of asthma diagnosis increased with the number of nasal symptoms, especially when they were associated with ocular symptoms, from 2.1% without rhinitis symptoms to 39.3% with three nasal complaints and 44.4% if nasal symptoms were accompanied with ocular symptoms. The fact that the association between rhinitis and asthma has been particularly notable in elderly patients with rhinoconjunctivitis may suggest an increased risk of asthma when there is greater likelihood of involvement of allergic mechanisms. Rhinoconjunctivitis was more frequent in elderly subjects with physician-diagnosed asthma with current treatment, compared to those without asthma treatment. Yet, as mentioned before, to confirm this hypothesis, objective biomarkers of specific immunologic mechanisms would be needed, which we did not perform. The ARIA classification of rhinitis provided important information with regard to the strength of the association with asthma, as it increased with increased persistency and severity of rhinitis, from mild intermittent rhinitis (OR 8.3, 95%CI 6.1-11.4) to moderate-severe persistent rhinitis (OR 39.9, 95%CI 27.5-58.0). While contradicting some previous studies[164-166], moderate-severe persistent rhinitis had an especially strong association with asthma, as also reported by other authors, in different age groups[23, 167-171]. Together, these results supported the extensive nose-lung interaction also in children and in the elderly.

Characterization of asthma-like symptoms in children in Portugal

The prevalence of asthma-like symptoms in children was 39.4% (95%CI 35.7%-43.3%). "*Waking during the night with cough*" was the most prevalent symptom (30.9%, 95%CI 27.5%-34.5%), followed by current

wheezing (i.e., "*wheezing in the last 12 months*"), with an estimated prevalence of 19.1% (95%CI 16.4%-22.1%). Wheezing prevalence was highest in children aged <6 years (31.0%, 95% CI 24.4-38.5) and significantly decreased with age. After the age of 5 years, most children with wheezing exhibited "*wheezing without a cold*" and "*wheezing with breathlessness*". Although no differences were found in the prevalence of current asthma in both genders, wheezing was more common in males (OR 1.96, 95%CI 1.30-2.97). These results suggested that age influenced asthma-like symptom patterns and were in agreement with other studies suggesting that wheezing in younger children was more closely related to immature airways and reduced airway caliber (particularly in young boys) and viral respiratory illnesses[172], whereas older children with wheezing tended to be symptomatic without concurrent respiratory infections.

In the ARPAkids study, the prevalence of current wheezing in children aged 3 to 5 years was 24.5% (95%CI 23.3%-25.7%), which was similar to our results. A multinational study of children aged 1 to 5 years living in Europe and in the United States of America showed that approximately one-third had wheezing, coughing or breathlessness[130], which was lower than our observation in children aged <6 years (47.9%). However, the time period considered in that study was only 6 months, while the time period in our study was 12 months. A study investigating the prevalence of asthma-like symptoms in New Orleans inner-city children aged 5 to 18 years found that 27.3% reported "*cough at night, apart from colds or chest infections*"[173]. Although no distinction was made regarding different causes of cough and associations to respiratory infections, the frequency of waking with cough in our study was rather similar (30.9%).

Thus, the prevalence of asthma-like symptoms in children was high, especially in younger ages. No statistically significant differences were found in the prevalence of asthma-like symptoms or current asthma according to region or any other analyzed socio-demographic variables.

Among children with current asthma, the majority had "*wheezing*" (76.5%, 95%CI 65.4%-84.9%) or "*waking during the night with cough*" (68.7%, 95%CI 56.6%-78.7%). These were the two most common reported symptoms, suggesting uncontrolled disease.

Characterization of rhinitis in the elderly population in Portugal

The most common nasal symptom in elders with current rhinitis was "*runny nose without a cold*" followed by "*sneezing and itchy nose*". The latest was the most reported symptom in the rhinitis study in adults[86]. Differences in nasal symptoms in the elderly may be expected, possibly due to concomitant age-related changes in nasal physiology in the elderly (nasal glandular atrophy, vascular changes with reduced nasal blood flow, decreased nasal humidification, decreased mucociliary clearance, and structural changes of the nose, including collagen atrophy and weakening of the septal cartilage)[174-177], which may contribute to more frequent watery rhinorrhea. A neurogenic dysregulation may be considered when topical ipratropium bromide is effective in reducing rhinorrhea in these patients[26]. In fact, it is likely that several mechanisms underlie the pathogenesis of rhinitis in the elderly, with potential interaction between inflammatory conditions and the influence of aging on nasal physiology[26, 178].

For the first time, rhinitis was classified according to ARIA in the elderly at the population level. We found that 49.1% of the elders had mild intermittent rhinitis, 7.0% mild persistent rhinitis, 27.5% moderate-severe intermittent rhinitis, and 16.4% moderate-severe persistent rhinitis. Rhinitis symptoms can become milder with age[178, 179]. However, in our study, more than 40% of the elders with rhinitis presented moderate-severe disease, showing a high impact of the disease among the elderly Portuguese

population. The overall rhinitis symptoms duration was of more than 25 years in the majority of the elders, showing a long-term disease.

Current rhinitis prevalence was higher than the national prevalence in Alentejo (57.7%) and Lisbon and Tagus Valley (35.7%), but both regions presented higher prevalence of intermittent rhinitis. Overall rhinitis symptoms and current rhinitis prevalence did not differ between elders living in rural or urban regions. However, those living in a rural region had a higher prevalence of moderate-severe persistent rhinitis. This was an unexpected result. Several studies have found that the prevalence of allergic rhinitis was higher in urban than in rural areas[180-182]. Moreover, in the previous study in adults[86], rural inhabitants presented significantly higher levels of intermittent rhinitis. It is possible that confounding factors, such as lifetime changes in the living region (from urban to rural regions and vice versa), may have contributed to these results, though the influence of other environmental factors could not be excluded.

Asthma and rhinitis in the extremes of life: substandard care?

Less than half of children with current asthma had been on inhaled controller medication in the previous 12 months, despite being symptomatic. Those who only used inhaled reliever medications were twice as likely to have experienced an "*asthma attack*" in the same time period. Despite the recommendation to perform lung function tests for the diagnosis and follow-up of asthma patients, only 52.9% of those aged ≥6 years had ever undergone lung function tests. Taken together, our results may suggest substandard asthma care in Portuguese children, especially in rural areas, where the frequency of lung function tests, allergy tests and used inhaled medication were lower.

Regarding rhinitis in the elderly, despite overall symptoms duration of more than 25 years in the majority of the elders and that more than 40% presented moderate-severe disease, only one third of patients with current rhinitis had been physician diagnosed and the same percentage had been under treatment for this disease in the previous 12 months. The association found between rhinitis and asthma further emphasized that overall rhinitis care may be substandard. About 40% of elderly patients with physician-diagnosed asthma had complaints of current rhinitis but were not diagnosed or treated for rhinitis. This is reinforced in those on current treatment for asthma and concurrent current rhinitis, since more than half of these patients had no physician diagnosis of rhinitis. Considering that rhinitis symptoms interfere with asthma control[6, 21, 22], there should be a greater awareness for the assessment of nasal disease in patients with an asthma diagnosis. The fact that a strong association was found between physician-diagnosed asthma, asthma on current treatment and all ARIA classes of rhinitis further supported the importance of an accurate rhinitis diagnosis in the elderly, including in milder presentations.

After adjusting for independent variables, living with relatives and especially living in a nursing home was associated with current rhinitis and also with physician-diagnosed rhinitis and asthma. This may suggest that these elderly subjects, compared to those who reside in their own house, could have greater frequency of rhinitis and asthma or better access to medical care. The fact that those individuals living in their own house had current treatment less frequently may either reflect better disease control in this group, or, on the contrary, support the assumption regarding worse access to medical care in elderly subjects living in their own house. Yet, it should also be considered that those elderly living in nursing homes often have greater deconditioning or multiple co-morbidities, which could contribute for disease misdiagnosis.

We also found regional differences across mainland Portugal. The greater difference between current rhinitis and physician-diagnosed rhinitis prevalence was observed in Alentejo. This may be related to the fact that the vast majority of elders living in this region had intermittent rhinitis (98.8%), but may also

reflect lower access to health care in this area. Alentejo is an inland region, also characterized by the highest absolute pollen concentrations in mainland Portugal, which occur seasonally, due fundamentally to the pollination of *Poaceae, Parietaria* or olive tree[183].

Despite our studies limitations, these results can thus support the need to implement new strategies for improving awareness management and control of these diseases, aiming at reducing rhinitis and asthma burden also in children and the elderly.

Definition of early childhood wheezing phenotypes related to asthma persistence

The high prevalence of asthma in Portugal, since very young ages, reinforced the need to accurately diagnose asthma cases early, with the aim to initiate proper treatment and to reduce the asthma burden since its very beginning. Wheezing was a major complaint in children with asthma, especially frequent at younger ages. The complexity of diagnosing asthma at early ages may arise from the very distinct long-term outcomes of early recurrent wheezing in children (author publication – full text available in the Attachments sections – number 1)[74]. Using data from our cohort study, we developed a multidimensional, "unbiased" statistical cluster model that combined different patient characteristics, based on questionnaires and skin prick tests, to assess the prognosis of asthma persistence in preschool children with recurrent wheezing. This prospective study allowed identifying three distinct early childhood wheezing phenotypes, which were predictive of asthma persistence, use of control treatments and lung function at school age and adolescence. Atopy and rhinitis were two independent risk factors for asthma persistence.

This study represented the largest cohort of children with recurrent wheezing conducted in Portugal so far. The "unbiased", hypothesis-free, multidimensional statistical modelling of prospectively obtained data was important to validate previous international phenotypic classifications in this distinct cohort. In order to reduce subjectivity, children with recurrent wheezing were included irrespective of any classifications or *a priori* definitions. The set of variables included in the cluster analysis was exclusively derived from the logistic regression model for asthma persistence, which included all study variables.

This study had the advantage of a long follow-up time (13 years). The children were systematically evaluated at key moments of childhood development up to adolescence. We analyzed data for a large number of patient characteristics, including respiratory symptoms, personal and family histories of allergic diseases, environmental exposures, skin prick testing, and lung function evaluations. All data regarding diagnoses were validated by Immunoallergy specialists.

The main limitations of this study were the loss of participants during follow-up and the lack of objective markers for environmental exposures (namely, tobacco smoke) and preventive treatment use. At the end of 13 years of follow-up, 55% of the cohort was lost to follow-up. However, the cohort remained homogenous in the main baseline characteristics throughout all evaluation time points.
Furthermore, the sample selection was based on the first visit to a specialized center, which may be associated with a bias. However, the sampling strategy provided sample homogeneity with respect to clinical manifestations and wheezing frequency and severity. Although atopy at preschool age was defined by skin prick test results to a broad number of possible clinically relevant set of aeroallergens and food allergens, these were not fully comprehensive and no other skin, blood or other test results were

considered. In some cases, allergen mixtures were used to limit the number of skin prick tests to perform in young children.

The identification of wheezing phenotypes based on multidimensional statistical modelling of data, without predefined criteria allows a less biased classification of wheezing. However, it is important to consider that the modelling will always depend on study design, definitions used and on the selected variables and clustering method. To be accurate, this modelling strategy is less biased (rather than "unbiased") than the traditional hypothesis-driven clustering. In order to further improve childhood wheeze classification, the time dimension should be taken into account, to cope with the recognized variability of asthma. The wealth of data from longitudinal datasets requires the application of flexible mathematical approaches to model the effects of time-varying factors (namely environmental exposures) and wheeze outcomes with multiple trajectories, measured at different time points. Modelling the effect of time-varying with time-invariant related factors and using the age at data collection as a continuous variable can be possible using statistical methods such as latent class growth analysis[184].

The "unbiased" phenotypic classifications derived exclusively from data can be complementary to groups defined *a priori* or based on directly observable criteria. The Tucson Children's Respiratory Study was one of the most relevant birth cohort studies, designed to determine wheeze risk factors[59, 65]. A total of 1246 new-borns were recruited and the cohort reflected a general population sample. Three wheeze phenotypes were defined: persistent atopic wheeze (corresponding to 15% of the whole sample, characterized by wheeze onset <3 years of age and persisting at 6 years), late non-atopic wheeze (14%, with wheeze absent at the age of 3 years but present at 6 years) and early transient wheeze (20%, with wheeze onset <3 years of age but absent at 6 years); 51% of the included children had no wheeze. These three wheeze phenotypes have been validated in independent cohorts, namely in France and in the United Kingdom, by unsupervised statistical methods[185, 186]. Other prospective studies using "unbiased" clustering proposed a classification with a higher number of phenotypes[60, 187]. Nevertheless, all these studies agreed that one of these clusters of children was characterized by having transient early wheezing. This phenotype has been associated with viral infections and lower airways caliber. Typically, there was no personal or family history of allergic or respiratory diseases. This phenotype has been linked to passive smoking and day-care attendance during the first year of life and having older siblings[65]. These children may exhibit early lung function limitation with airway obstruction pattern that tend to improve overtime[65, 71]. Consistent with this description, a non-atopic transient wheezing phenotype was also found in our cohort, which was associated with not having atopy, rhinitis or atopic dermatitis and no family history of asthma, despite early onset of wheezing (<24 months). In our cohort, this phenotype was also associated with day-care attendance during the first year of life. Therefore, children with >3 episodes of wheezing dyspnea responsive to bronchodilators and symptom-free intervals between exacerbations at preschool age can become symptom-free, especially when no other risk factors exist. Notwithstanding, most children in our cohort remained symptomatic throughout the study, in contrast with the Tucson study that stated that 60% of preschool children with wheezing became asymptomatic by age 6 years[188]. This difference was most likely due to the distinct sample selection criteria. In the present study, we selected children with at least three wheezing episodes over the last 12 months, but the Tucson study only required one episode[188]. However, we could not exclude the possibility that, in our study, full symptom resolution may have occurred more frequently among the children lost in follow-up.

Opposite to the non-atopic transient wheezing phenotype, the persistent atopic wheeze phenotype was characterized by a later onset of wheeze (>24 months) and personal and family history of allergic multimorbidity, as previously described[59, 65, 189, 190]. This allergic multimorbidity phenotype had the worst prognosis related to symptoms persistence in adolescence. Impaired lung function was more common than in the non-atopic transient wheezing. It is important to note that despite the different proposed classifications of childhood wheeze phenotypes among the different studies, authors have agreed that

atopy was related to symptoms persistence[60, 65, 185-187]. In our study, atopy at preschool age was an independent risk factor for asthma persistence in adolescence. This reinforces the need to perform allergy tests (namely skin prick tests with allergen extracts) at early ages. Allergen sensitization in symptomatic children should strengthen the diagnosis of asthma (although atopy is not mandatory for the diagnosis)[6, 191], and interventions that reduce allergen exposure may be associated with a more favorable prognosis[192].

Lastly, similar to the non-atopic asthma phenotype described by the Tucson group[65], we have identified a third wheezing phenotype that was persistent, yet not associated with systemic atopy. This phenotype was less frequent than the atopic persistent wheeze phenotype (only 14% of children from our cohort were included in this group) but it was also characterized by worse prognosis regarding persistent symptoms, medication use and impaired lung function. Interestingly, although less usually described, such a phenotype has also been found in other cohorts[65, 185, 186, 189, 190]. Nonatopic persistent wheeze has been associated with respiratory infections-related airway hyperreactivity. In our cohort, despite an early onset of wheeze and the absence of systemic atopy, these children differed from the group with good prognosis in the fact that most had rhinitis. Furthermore, most children had family history of maternal asthma and day-care attendance during the first year of life was less frequent. Therefore, unlike previous reports[193-196], we found that, independent of atopy, rhinitis at preschool ages was a significant risk factor for asthma persistence. Local allergic rhinitis may be involved, defined by the presence of IgE-mediated allergy that is only evident in the nasal mucosa, without positive skin prick tests or detectable specific IgE in the serum[26, 27, 197, 198], but we did not test these children for local IgE-mediated allergic responses. Notwithstanding its high prevalence in preschool children (estimated to be 43% in Portugal), rhinitis remains underdiagnosed particularly in children[199]. Among preschoolers, the severity of wheezing and rhinitis are strongly associated[61]. Thus, our results emphasized the need to value and assess nasal symptoms in children, independent of atopy, since early ages. Taken together, these results further strengthen the need to consider the rhinitis and asthma multimorbidity in clinical practice and also in research.

Preschool eczema was also associated with asthma persistence, as consistently reported[59, 191, 195, 200]. This inflammatory disease is another feature of the allergic multimorbidity phenotype. However, we found that preschool eczema was not an independent risk factor for asthma persistence. This may have resulted from the association between eczema and rhinitis and/or atopy. The Asthma Predictive Index includes eczema as a major criterion and rhinitis as a minor criterion for predicting asthma persistence[200]. In contrast, in our study, we identified rhinitis, not eczema, as an independent risk factor for asthma persistence.

Asthma prevalence during school ages and adolescence was higher in children with a maternal asthma history. However, there was no association with paternal asthma. Although parental asthma diagnosis was only self-reported in the present study, results of other studies indicated that maternal and paternal characteristics may have distinct impacts on offspring asthma development[201, 202]. In particular, the maternal influence on gene-environmental interaction was greater than paternal influence regarding IgE production and asthma development. Exclusive exposure to maternal environmental factors during fetal development, fetus-maternal shared perinatal environment exposures (including breastfeeding), different hormones and genomic imprinting are possible explanations for the mother/father different impacts on asthma development of their offspring[203].

Children who attended day care during infancy had significantly fewer persistent symptoms. However, this “protective” effect seemed to be lost with age; no effect was observed in adolescence. Also, day care attendance frequency was similar among the worse prognosis atopic persistent and the better prognosis nonatopic transient phenotypes. This suggests that the potentially protective effect of day care

attendance at early ages may depend on other characteristics of the children in each group. This hypothesis may be consistent with previous studies that demonstrated that the protective effects of day care attendance with respect to allergen sensitization and respiratory symptoms depended on genetic variants[204]. In two independent unselected birth cohorts from distinct geographic areas, the association between day care attendance and sensitization/atopic wheezing appeared dependent on a genetic variant in toll-like receptor 2 gene. Day care attendance was protective to those children with the T allele for toll-like receptor 2/-16934, whereas in those who were AA homozygotes the association tended to be in the opposite direction[204]. In complex diseases such as asthma, genetic predisposition may need to be taken into account when assessing the effect of environmental exposures and not all individuals may benefit from a specific intervention.

Exposure to maternal tobacco smoking during pregnancy is a major concern, and it has been associated with asthma in children in several studies[201, 205-209]. Despite a significant trend over time that showed a decreasing frequency of asthma in children without maternal smoking during pregnancy, our results could not confirm that association. Similarly, we found no association between environmental tobacco exposure and asthma persistence. However, it is important to stress that, in this study, exposure to tobacco smoke was only self-reported; it was not objectively measured, and, thus, the results may be significantly biased.

Lung function abnormalities were associated with asthma persistence in adolescence. Nonatopic persistent wheezing had the worst prognosis in lung function parameters during school age and adolescence. Our results indicated that children with early wheezing onset were at risk of asthma persistence and lung function impairment when associated diseases were present, namely rhinitis, or when there was a family history of maternal asthma, even in the absence of atopy. Cohort studies have acknowledged atopy as a determinant of lung function[193-195, 210], but rhinitis alone, without atopy, has not been reported. However, a recent publication demonstrated that lung function trajectories were associated with asthma combined with comorbidities rather than with allergen sensitization[209]. Assuring adequate lung function development throughout each child's growth is critical. Early childhood-onset wheezing that persists into adolescence represents the clearest target group for interventions to maximize lung function outcomes[211].

Biomarkers in allergic rhinitis and asthma multimorbidity in children

The previous studies further extended the data on the association between rhinitis and asthma. Children with the worst prognosis of asthma persistence until adolescence were the ones with allergic rhinitis and asthma multimorbidity, reinforcing the need for integrated clinical approaches.

Integrating care pathways with biomarkers – adding nasal function in the concurrent evaluation of allergic rhinitis and asthma control?

Integrated care of patients with concurrent rhinitis and asthma is essential at the clinical level[6, 7, 22]. The subjective assessment of symptoms is crucial to evaluate severity and control of rhinitis and asthma multimorbidity. Objective markers complement this subjective assessment. Lung function parameters support asthma diagnosis and future risk assessment, and are currently recommended to be performed regularly with the aim of monitoring and maintaining the best possible values for each patient[6, 7].

Likewise, objective measures of nasal function have been recommended[84, 85], and successfully used for the objective evaluation of rhinitis and its control[89-92], including in children[93, 94].

In our exploratory study, we analyzed the PNIF of healthy children and children with allergic rhinitis and asthma multimorbidity, which is the simplest validated method to assess nasal function[84, 85]. We found an anthropometric independent correlation between PNIF and PEF and FEV_1 in school-aged children (6 to 12 years). Our results suggested that, ideally, at least PEF values should be considered, besides age and gender, when evaluating nasal function by means of PNIF in this age group. Furthermore, this study strengthened that the objective measurement of nasal flow and subjective symptoms scores may not be correlated. We described, for the first time, no associations between PNIF and the CARATkids questionnaire scores, except for nasal obstruction self-report in the past two weeks and PNIF expressed in z-scores in children with allergic rhinitis and asthma. Therefore, these results suggested that, similarly to lung function parameters in asthma, PNIF can provide complementary objective information to subjective control assessment in children with allergic rhinitis and asthma multimorbidity.

We hypothesized that PNIF was associated with PEF and FEV_1, independent of anthropometric variables. This had not been published before in this age group but it is critical for an accurate interpretation of PNIF values in children. Sequential upper and lower airway standardized evaluations of the same individual were performed, reporting PNIF values before and after nasal topical alpha-adrenergic use and PEF and FEV_1 values before and after inhaled $beta_2$-agonist. A balanced sample of patients comprising different asthma control levels was studied, together with age and gender-matched healthy controls. Consistent correlations between upper and lower airway flow were found. These correlations persisted after adjusting to age, gender, height and weight, suggesting that the association between upper and lower airflow measures is independent and not a simple reflection of children's growth or body size. The correlations found between PNIF and PEF expressed in z-scores (which is an age, gender and height-independent variable) also supported our conclusions.

This study's main limitation was its cross-sectional and exploratory design. Further studies with larger heterogeneous samples are needed but our results suggested the need to include at least PEF, besides age and gender, in these future studies concerning PNIF reference equations in this age group. Our study did not include children with rhinitis or asthma alone. Most children with asthma have rhinitis and we did include asthmatic children with different levels of disease control and lung function tests results, but the inclusion of children with asthma or rhinitis only is relevant for generalization of the results to these groups of patients. Another limitation is the lack of children of other ethnicity, which limits our results only to Caucasians. Despite the fact that all diagnoses were validated by medical specialists (based on anamnesis, clinical files and medical examination including anterior rhinoscopy), no nasal endoscopy or imaging techniques were performed in this study. Therefore, we could not firmly rule out adenoid hypertrophy or nasal septum deviation (which could introduce biases into our study), but only excluded children with known or suspected diagnosis or with observable anatomical nasal abnormality on anterior rhinoscopy.

The fact that nasal flow can be influenced by lower airway status has long been a concern[85]. Studies in adults have suggested an independent correlation between PNIF and PEF[100], or FEV_1[101]. Although initially considered a limitation of PNIF, the concept of a single disease of the airways in rhinitis and asthma multimorbidity has changed this view, and the impact of the lower airways is now taken into consideration in the study of nasal function[85, 212]. The correlation between PNIF and PEF has been reported in healthy children[98], but no multivariable analysis including PEF values was reported and FEV_1 wasn't evaluated. As previously described[96-98], we have found a correlation between PNIF and age, height and weight. PNIF values were higher in boys compared to girls but, as reported by Papachristou et al, this difference did not reach statistical significance until the age of 12 years[96]. Since age was the only variable consistently reported in the literature to be correlated with PNIF[95-99], multivariable linear models

for PNIF using this variable were presented. We chose to include baseline PEF rather than FEV_1 in the final model since baseline PEF is more easily obtained, even with only a peak flow meter and without the need to use bronchodilators. After accounting for these variables, there still remains a large degree of variability in PNIF values, suggesting that further variables, such as anatomic variations or possibly nasal inflammation may refine the modelling of data.

Using acoustic rhinometry (before and after alpha-agonist) and spirometry with bronchodilation test, Chawes and collaborators found an independent and consistent correlation between nasal volume and FEV_1 in children aged six years[213]. In accordance to our results, the correlation between upper and lower airways measures were observed independently of rhinitis and asthma diagnosis[213]. This suggests that the independent correlation is a consistent finding in healthy and asthmatic children. Our results further support the hypothesis that the correlation between upper and lower airways may reflect a physiologic background for the common rhinitis and asthma multimorbidity[213].

Pathologic mechanisms could also be involved. A continuous nasobronchial inflammation process has been described in rhinitis and asthma[21, 23]. However, we found no association between PNIF and FeNO levels or between reversibility of the upper and lower airways, also in agreement with Chawes et al findings[213]. We also found no differences in PNIF according to multiple sensitization to aeroallergens, as described before[213, 214]. Nevertheless, Chawes and collaborators reported an association between blood eosinophil counts and nasal eosinophilia with nasal patency[213], which we did not evaluate.

Reference values for the pediatric population have been published, all measured in non-decongested noses[95-99]. Yet, it is important to measure PNIF values before and after decongestion to elicit the role of mucosal swelling[84, 85]. Our baseline PNIF values were similar to the reported values in healthy children in Brazil[97, 98]. Prescott et al and van Spronsen et al reported slightly lower PNIF values[95, 99], while Papachristou et al, who analyzed the largest sample of healthy children, reported higher PNIF values[96]. The discrepancy between study results could be due to different studied populations and to different methods (for instance, not performing the PNIF maneuver from the residual volume until reaching total lung capacity[95], or PNIF values collected while sitting[95, 99], or standing up[96-98]). A recent study in adults showed a trend towards a positive effect of the standing position on PNIF, although not statistically significant[215]. To our knowledge, the effect of body position on PNIF in children has not been analyzed. Papachristou et al published PNIF values were similar to our observed decongested PNIF values[96], which we used as a reference.

Data regarding the association between CARAT questionnaires and objective measures of nasal function did not exist before this study. Nevertheless, previous studies with children have found an agreement between objective and other subjective measures of nasal obstruction[93, 216], while other researchers reported the opposite[217]. The reason for this disagreement in multiple studies is probably multifactorial. For instance, given the known influence of other variables including age, gender and anthropometric variables in upper airway function in children, it is important to use reference values to obtain predicted percentages or ideally z-scores to interpret the results. We found an association between PNIF expressed in z-scores (for age and gender) and nasal obstruction self-report in the CARATkids questionnaire in children with allergic rhinitis and asthma. This association was also independent of PEF. Evaluating each nostril individually instead of bilateral measurements has been shown to allow stronger correlations between objective and subjective measurements in adults[87], and also described in children using rhinomanometry[217]. On the other hand, the subjective scoring tool influences the results. Scales with fewer score options seem to increase the probability of an association between objective and subjective measurements[218]. The exact questions and time of symptoms evaluation may also affect this association. In our study, we didn't find statistically significant differences in PNIF comparing healthy children and children with rhinitis and asthma reporting nasal obstruction in the CARATkids questionnaire. This needs to be analyzed in future studies with larger samples, but it could be influenced by the fact that

CARATkids questionnaire considers nasal symptoms self-reported by the children regarding the last two weeks and not necessarily current symptoms at the time of PNIF measurement. Moreover, in a previous study in adults, patients with asthma significantly rated their nasal obstruction by visual analogue scale more seriously than non-asthmatic controls with comparable PNIF values[101]. Apparently, the sensation of nasal obstruction in asthmatics may be different from controls despite being in the same PNIF group[101]. On the opposite direction, children on long-term treatment for chronic rhinitis may underreport the amount of nasal congestion[219], and it has been reported that children may be more accepting of mouth-breathing than adolescents[218]. Last but definitely not least, children might also be influenced by their parents or guardians' perceptions during the subjective assessments, which may also contribute to this disagreement[217]. Therefore, as in asthma, objective and subjective assessments appear to evaluate different parameters that may not be directly related, and PNIF may provide complementary information to the subjective evaluation of rhinitis and asthma in children. Since the subjective feeling of nasal obstruction may be valued differently by individual subjects, future research studies addressing PNIF in children with rhinitis and asthma may take advantage of additional comparisons with other measures of nasal patency, namely using the "golden standard" rhinomanometry.

"Unbiased" molecular phenotyping – contributing to the link between clinical phenotypes and disease endotypes?

According to the current paradigm, asthma should not be regarded as a single disease, but rather as a complex syndrome. The "allergic asthma" (as defined by GINA) is a classic form of persistent asthma, with a typical childhood onset, accompanied by allergic features, including sensitization to allergens and allergic rhinitis[6, 220]. Cluster analyses of asthma phenotypes also support the existence of childhood onset "allergic asthma" as a distinct endotype[28, 29, 220, 221].

Our case-control study explored the potential of untargeted metabolomics to uncover "unbiased" differentiating metabolic features of allergic rhinitis and asthma multimorbidity in children. Using NMR exploratory metabolomics, differentiating subsets of NMR spectral areas in urine and saliva were found. Some of these metabolic variables were associated with such clinical readouts as lung function parameters, FeNO levels and multiple allergenic sensitization. The identification of the metabolites contributing to these differentiating variables further supported several hypotheses, namely regarding the role of arginine, taurine, quinolinate and butyrate in allergic rhinitis and asthma multimorbidity. Our results require validation and these hypotheses will need further analyses.

At variance with most of the previous metabolic profiling studies, we used untargeted metabolomics to uncover "unbiased" differentiating metabolic features of a specific clinical phenotype and considered allergic rhinitis and asthma multimorbidity for the first time. Furthermore, we have used two non-invasive samples, collected consecutively from each child, aiming for a more "systemic view". A balanced sample of patients comprising different asthma control levels was studied, together with age and gender-matched non-atopic healthy children. All diagnoses were validated by specialist medical doctors and all children were characterized regarding airway inflammation measured by FeNO and lung function with spirometry and bronchodilation test. Spectral NMR areas of urine and saliva were associated with allergic rhinitis and asthma using statistical analyses that involved a pre-selection of variables followed by linear modelling. Compound identification was performed, to benefit possible methodology validation, as well as raising pathophysiological hypotheses.

Molecular profiling by untargeted approaches may provide novel insights to characterize clinical phenotypes at the molecular level, independent of predefined sets of hypothesis-driven metabolites. This

strategy may bring complementary data to our current knowledge and to the results of hypothesis-driven studies (author publication – full text available in the Attachments sections – number 2)[104]. Similarly to the "unbiased" clustering methods discussed before, it is important to acknowledge that untargeted metabolomics results will always depend upon the participants' inclusion criteria, study design, samples procedures and on the analytic technique and its intrinsic analytic coverage. With the inclusion of a high number of patients in heterogeneous populations, these untargeted "omics" analytic technologies can be used to find relevant molecular clusters and potentially contribute to improve our classification of the diseases. However, exploring the molecular profiling of a clinically defined asthma phenotype may reduce heterogeneity and potential bias. In fact, one probable justification for the distinct results obtained so far by the existing metabolic profiling studies in asthma may rely on the distinct study populations, especially considering the heterogeneity of asthma[222].

The "allergic asthma" phenotype is the most commonly recognized asthma group in childhood. Although this is the best described asthma phenotype, a curative treatment is lacking and still heterogeneity exists in patients' clinical presentation, disease severity and response to therapy[6, 223]. Atopy is an important feature that explains some but definitely not all the mechanisms involved. Apart from the involvement of the adaptive immune responses to allergens in a typical Th 2 and IgE-driven disease, more recent insight also highlighted a major involvement of innate lymphoid type 2 cells, producing type 2 cytokines in response to other common triggers such as pollutants and viruses that coexist and interact with allergens[224]. The view of eosinophilic asthma as an exclusive allergic Th2 or non-allergic innate lymphoid type 2 cell disorder (and even the exclusive neutrophilic Th1 and/or Th17 disorder) represents an oversimplification. It is important to consider the dynamic overlap of mixed cell types and cytokines contributing to the disease that occurs in each asthma patient[75].

Exploratory metabolomics aims to provide a global snapshot of all small-molecule metabolites in biospecimens, free of observational biases inherent to more focused studies of metabolism. A hallmark of metabolic fingerprint is the use of multivariable analysis to identify class differences in highly complex datasets. The Principal Component Analysis (PCA) is the most commonly used statistical method. Spectral features contributing most to data variation or separation are identified for further analysis. The unsupervised nature of the PCA algorithm only reveals group structure when within-group variation is sufficiently less than between-group variation. In our study, the first two components of the obtained PCA model explained only 17% and 33% of the variation of the urine and saliva data, respectively. Neither rhinitis and asthma, nor asthma control contributed to the main sources of variation of the data in both urine and saliva models. This may be due to the fact that the broad range of the metabolome that can be captured by metabolomics profiling is sensitive to both internal and external influences that may be unrelated to disease status. It could also reflect heterogeneity in allergic rhinitis and asthma-related metabolic pathways or even still limited analytical coverage or discriminatory capacity to identify global shifts in multiple correlated metabolites. Yet, this does not mean that there are no variables of interest to differentiate asthma in these biospecimens. In fact, similar findings have been described by the different research groups using different biospecimens including urine, saliva and other samples such as plasma and EBC in multivariable analyses in asthma profiling studies[110, 121, 123, 225-229]. Feature selection can allow for the extraction of important metabolites[107]. This is applied to: a) reduce overfitting, b) improve model performance and c) gain a better understanding of the relationship between the metabolic features and the response clinical variable. Several feature selection strategies have been used by different research groups to identify metabolic discriminatory features classifying asthmatics from healthy controls. For instance, metabolites summary scores have been created based on spectral variables shown to be associated with asthma status by removing spectral variables listed as being lower in significance on the variables of importance plot until a pre-set false-positive rate for healthy individuals[225, 226], or until a maximum goodness of prediction were reached[123]. We chose univariable spectral area selection to measure the importance of each variable individually on rhinitis and asthma in a simple and easy to

interpret less biased approach[227, 229-231]. These preselected sets of variables were further reduced by linear modelling and logistic regression was applied to select the final best set of spectral areas. As response variable, comparisons were made between healthy children and children with partly controlled and uncontrolled asthma, since more significant differences were expected to be found than in controlled asthma. Since partly controlled and uncontrolled asthma may be metabolically heterogeneous themselves, separate models were built for each group. In fact, by using this approach, different subsets of spectral areas and different metabolites were identified that differentiate the two groups, except for pantothenate which was identified in urine as a relevant differentiating metabolite in both models. Distinct from previous metabolic profiling studies in asthma, we analyzed saliva and urine simultaneously from the same subject, but found distinct differentiating metabolites subsets in the two samples. However, some of the identified metabolites were indeed common to previous metabolomics profiling studies and have been linked to asthma. In particular, arginine and fatty acid metabolism are common reported pathways in asthma metabolomics studies, regarding both experimental animal models of allergic airway inflammation[232-235], and clinical studies in humans[116-118, 120, 122, 225] (author publications – full text available in the Attachments sections – numbers 3 and 4)[105, 106].

An enrichment of pathways reflecting increased metabolism of amino acids in asthma has been anticipated[108]. Amino acids are mediators of immunological activities in asthma and may have antioxidant functions, namely taurine[115, 116]. Urinary citrate has been previously reported to reduce during asthma exacerbations[236]. Low systemic arginine levels have been described in asthmatics[118, 237]. Decreased levels have been associated with induction of arginase activity in asthma, an enzyme that can be induced by type 2 cytokines[238, 239]. Arginine can also act as a substrate for nitric oxide production[240], but we found no association between salivary arginine and FeNO levels. In fact, increased levels of FeNO in asthma could reflect metabolism of arginine from a compartmentalized pool in which arginine content is not reflected by systemic arginine levels, as supported by arginine increased levels in EBC and pulmonary tissue[117, 241]. Other arginine-independent sources of FeNO need also to be considered, including thiol-containing biomolecules (such as S-nitrosoglutathione) and nitrite releasing nitric oxide due to airway acidification[242, 243].

Although we found that these amino acids discriminated asthmatics, the link with the reduced level of free metabolites in the saliva and asthma is unknown. This could be related to systemic levels but also to dietary changes or salivary gland secretion. Of relevance, taurine is the most abundant free amino acid, mainly in proinflammatory cells and tissues exposed to elevated levels of oxidants, such as the salivary gland, where it exerts a role in regulation of salivary flow. Altered salivary flow rates have been described in asthmatics, which can contribute to changes in saliva composition[244, 245].

Quinolinate is an endogenous metabolite of tryptophan at the kynurenine pathway, which has been linked to inflammatory pathways[246, 247]. Quinolinate may also derive from alanine, aspartate and glutamate metabolism and be involved in nicotinate and nicotinamide metabolism, precursors for generation of coenzymes nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate. These coenzymes are crucial in many metabolic pathways including glycolysis, tricarboxylic acid cycle and fatty acid metabolism. The synthesis of nicotinate from tryptophan is possible but it is a slow and largely inefficient process. Quinolinate levels were found to be higher in serum and lower in EBC in adult patients with allergic asthma[248]. Moreover, systemic quinolinate has been associated with eosinophilic cation protein and eosinophils in bronchoalveolar lavage fluid, and peak asthma symptom scores after rhinovirus challenge[248]. Interestingly, besides a negative correlation with lung function parameters, we also found a positive association between urinary quinolinate levels and multiple allergenic sensitization and FeNO levels, which could further support quinolinate as a marker in the type 2 inflammatory pathway[248]. However, the pathophysiologic mechanisms of enhanced systemic quinolinate in asthma remain to be determined. Urinary tryptophan has been also identified as a discriminatory metabolite in asthmatic

children[225]. The immunomodulatory function of indoleamine 2,3-dioxygenase, the rate-limiting enzyme in tryptophan catabolism, and other compounds generated during tryptophan metabolism have also been described in asthma, supporting that further insights on this metabolic pathway may contribute to increased knowledge in asthma[115, 246, 248-250].

Butyrate has also been identified in exploratory metabolomics studies in asthma[120, 122]. This molecule is a short chain fatty acid, mostly produced in humans in the gut by anaerobic fermentation of undigested carbohydrates and fiber polysaccharides. Besides its role in the energy requirement, butyrate is suggested to be a mediator in the communication between commensal microbiota and the innate and adaptive immune system, with multiple anti-inflammatory effects, including suppression of nuclear factor kappa B activation and interferon gamma production, upregulation of peroxisome proliferator-activated receptor gamma and promotion of peripheral regulatory T cell generation, affecting effector cells migration, adhesion, proliferation and apoptosis[251-255]. It has been recently reported that butyrate can influence type 2 innate lymphoid cells proliferation and function, associated with reduced airway hyperreactivity and inflammation in mice[256]. The principle mechanism of action involves epigenetic regulation through the inhibition of histone deacetylase[251]. Overall, histone deacetylase inhibitors have been recently considered in the treatment of asthma and other inflammatory lung diseases[257]. The lower levels of butyrate were associated with partly and uncontrolled asthma and impaired lung function. Taken together, exploring these findings may bring additional evidence to the role of gut microbiome in allergic rhinitis and asthma[127, 258, 259].

In our study, using the identified differentiating spectral regions subsets, the obtained summary scores showed good classification accuracies. While some metabolites associated with allergic rhinitis and asthma in our study are biologically plausible and supported by previous experimental data, these preliminary results require validation. This step is critical to assess true discriminatory ability and support for accuracy of the reported biomarkers in independent cohorts. Furthermore, essential steps relate to monitoring external factors that may affect the metabolome and introduce significant bias to the results. This is important in any study design, especially when using such multivariable analytical techniques as NMR or mass spectrometry. External factors known to affect the metabolome include the age, gender, body mass index and circadian rhythm. Despite being well matched demographically (age, gender, body mass index) and all samples being collected at the same time of the day, it is important to highlight that diet and current asthma treatment may have interfered with the urine and saliva metabolic profile in our study. Although care has been taken to refrain from drinking or food intake before samples collections and most medication was inhaled and had been stopped for at least 12 hours, we cannot firmly rule out that our results were not affected by diet or medication. Nevertheless, no association was found between any differentiating spectral area and self-reported specific food intake or fasting time or drug use on the previous week, except for oral montelukast, which was associated with the variable comprising arginine and taurine in saliva. However, an explanation for this association is unknown to us, considering that montelukast had been taken more than 36 hours before saliva collection. Randomized trials and animal model studies analyzing the effect of medication on the metabolic profile will help distinguishing the effect of drugs from the disease effect on the metabolome[106]. After accurate validation of the results, we should be able to contribute to the rather incomplete and imprecise figure of relevant metabolic pathways in asthma pathogenesis (Chapter 1 – Figure 6), namely in the more specific clinical phenotype of childhood allergic rhinitis and asthma multimorbidity. Last, but definitely not least, while aiming to uncover differentiating features of a given disease or state, the determination of specificity of a given potential biomarker also needs to be analyzed. It is important to consider that many metabolites that have been reported to be associated with asthma in exploratory metabolomics have also been found in other diseases, namely cystic fibrosis, acute respiratory distress syndrome and even multiple malignancies[108]. While this does not negate their possible involvement in asthma pathogenesis, it questions their role as stand-alone asthma biomarkers.

We further assessed potential exogenous contaminants in EBC exploratory metabolomics analysis. Endogenous and microbial components in exhaled breath have the potential to contribute to the metabolic profiling of asthma. However, components in exhaled breath also originate exogenously[113]. Potential misinterpretation and inconsistency between study results may occur if the origin of certain metabolites in EBC is assumed as endogenous and thus considered useful biomarkers in asthma, despite the possibility of being originated exogenously, namely from the environment. Eliminating metabolites originated in the inhaled air from EBC can be particularly challenging and complex. According to recommendations, control experiments in which subjects inhale filtered or specific air mixtures that do not contain the specific compounds, or their precursors, that will be measured should be considered[112, 113]. This strategy may be insufficient given all the possible interactions of different air compounds with the molecules of interest, and it is not feasible in untargeted "omics" approaches where no compound preselection is done before data analysis. Despite the recommendations to address potential environmental contamination, this has not been routinely reported in many EBC metabolic profiling studies. However, based on the results of our study comparing air samples with human EBC samples, we reinforce the need to report ambient air controls during EBC samples collection and address the influence of exogenous contaminants.

In a seminal paper on NMR profiling of EBC, Bertini et al used a homemade EBC collecting device[260]. Humidified room air was pulled into the condensing apparatus and the analysis of this sample resulted in NMR spectra free of ambient air or device contamination signals, while an EBC metabolic profile was found[260]. However, the need to control for ambient air in NMR-based studies had been previously described. Using the standard commercial device EcoScreen®, Izquierdo-Garcia et al have reported the presence of a number of exogenous compounds in the room air samples, including, among other components, acetate, formate, propionate, lactate, benzoate, glycerol and propylene glycol[261]. In our study using a different standard commercial device (R-Tube®), the composition of the room air spectrum was similar to the one from EBC. Several reasons could be considered to explain our results, from ambient air contamination to the effects of the condensing equipment and samples storage or processing methodology. Although the influence of each of these factors has not been analyzed individually, our spectra were similar to Bertini et al published EBC spectra, using similar analytic procedures[260]. Furthermore, the RTube® collection system is made of inert, polypropylene materials and a silicon rubber one-way valve, and the NMR spectra of water from this collecting system has been shown to be clearly different from EBC spectra[262]. These strengthened a possible contamination of room air and EBC samples in our study. A number of possible factors could have contributed to the contamination, namely the limited space in the collection room, insufficient room ventilation and the exhaled breath of the assisting personnel. The need to control ambient air may be especially relevant when portable EBC collection systems such as the one we used in our study, are utilized in different environments, namely at patients houses, schools or work places. A case-control study design under standard procedures has been pointed as a solution that could minimize the effect of potential environmental contaminations, assuming that these should be randomly distributed between the groups of interest[229]. However, NMR spectral signals from ambient room air collected in the same place on different days and time of the day have been reported to be significantly different[261]. Unless all EBC collections were performed simultaneously, the case-control design may not be sufficient to guarantee homogeneity considering ambient air variability. Validation of findings with an independent cohort of cases and controls also minimizes the effect of potential confounders, but still requires confirmation in different environments. Thus, other approaches need to be considered during EBC collection. Subtracting the air background from the breath signal is a method that is being increasingly applied in gas-chromatography coupled with mass-spectrometry breath analysis, by measuring the alveolar gradient, i.e., the relative abundance of the metabolites in exhaled breath and in ambient air. Data interpretation is based on the assumption that equilibrium exists for all metabolites between the body and ambient air. This is difficult to apply in NMR-

based metabolomics data, in particular given the high correspondence of both spectra, as previously reported[261]. A common limitation of NMR is its relatively low sensitivity compared to mass-spectrometry based techniques. Currently, metabolites with concentrations lower than 0.5-1.0µM will usually be below the detection limit. Because of the difficulty of deconvoluting the peaks to quantify individual components, NMR metabolomics are often performed in a non-targeted fashion[263]. These can make discriminating exogenous from endogenous metabolites in EBC more difficult. Another approach is to consider fractioned EBC sampling. Commercial devices are currently available that allow the collection of different EBC fractions according to a pre-set threshold volume into upper/lower airways or airway/"alveolar" fractions. Based on exhaled carbon dioxide profiles, collecting only late-expiratory or end-tidal breath involves discarding the initial portion of exhaled breath (estimated dead space, phase I of exhaled breath, where carbon dioxide levels are low) or both dead space and transition phase (phase II) of exhaled breath, respectively. This collection method aims at a greater relative contribution of endogenous compounds in the resultant samples[264]. However, this method is not standardized. Concerns regarding reproducibility also arise due to distinct physiological properties of individuals including cardiac output besides pulmonary ventilation, breathing pattern and expiratory flow rate, which may alter metabolites concentration. So far, no qualitative differences were observed between the EBC fractions, although distinct metabolite concentrations were found[264, 265]. Moreover, airways metabolites may be lost when analyzing only a fraction of EBC and thus end-tidal breath analysis may not be suited for investigating airways diseases[266].

The use of a filter applied to the EBC collection system has also been tried[117, 120, 261]. Izquierdo-García et al placed a trap for room air contained water-soluble organic compounds in the collecting system. Although most of the spectral air signals were removed, NMR signals were still visible[261]. Motta and colleagues used a respiratory protection particulate filter while collecting room air and EBC using two devices: EcoScreen® and TURBODECCS®. With this strategy, the NMR spectra of condensed room air were devoid of signals, independent of the sampling device, while several NMR signals were reported in EBC samples[117, 120]. Thus, the use of high efficiency filters against particles with approximately 0.3µm mass median aerodynamic diameter might be a solution to address ambient air contamination in NMR-based studies, which deserves further analysis to check external validation in different environments. However, this provides no insight into the composition of room air inhaled by a patient. Another possibility would be the use of a standard air source during EBC collection. Although variable interaction between this source of air and EBC is inevitable, this procedure would allow standardization and independence from room air variability. Still, a sensitive analytical method that allows the distinction of exogenous from endogenous metabolites is necessary. In summary, our results reinforce the need to report ambient air controls collected simultaneously with EBC in metabolomics studies. A careful assessment of any potential contamination arising from ambient air or sample collecting and processing procedures is essential and NMR may not be adequate to discriminate exogenous from host and microbiome-originated metabolites using an alveolar gradient-like approach.

Chapter 6: Conclusion and Future Perspectives

The population-based nationwide epidemiologic studies were important to contribute with estimates on asthma prevalence and analysis of its association with rhinitis in two internationally data-lacking population groups: the children and the elderly. As an overall conclusion, these studies supported that asthma was a common disease, frequently associated with rhinitis, in both age groups. Our results further reinforced the need for a greater awareness towards asthma and rhinitis in a clinically integrated, global multimorbidity assessment, also in children and in the elderly.

The prospective cohort study supported the heterogeneity of recurrent wheezing in preschool aged children with distinct long-term outcomes. The data-driven defined preschool wheezing phenotypes included combined features from other previously described phenotypic classifications. Atopy and rhinitis at preschool ages were assigned independent risk factors for asthma persistence in adolescence. Early wheezing in children with associated diseases, particularly rhinitis (with or without allergen sensitization), tended to predict a worse prognosis of symptoms persistence and lung function impairment in later childhood and adolescence. Our study results favored that wheezing phenotypes identified at early ages from simple measurements could predict asthma persistence and lung function outcomes, which indicated the need for distinct management approaches.

A proactive integrating strategy to assess and control asthma together with rhinitis demands for biomarkers in the multimorbidity context. The objective evaluation of nasal function can reach global dissemination in clinical practice, using simple, non-invasive methodologies such as PNIF measurements. Our study extended the data supporting PNIF as complementary objective information to subjective allergic rhinitis and asthma control and contributed to suggest the need to consider, at least, age, gender and PEF when interpreting PNIF in school-aged children. The physiologic background for the common rhinitis and asthma multimorbidity was further reinforced.

Aiming to characterize the rhinitis and asthma multimorbidity at the molecular level, an untargeted metabolomics approach by NMR in non-invasively collected samples was explored. Several setbacks were found, mainly regarding EBC profiling, which is known to be a highly variable matrix and is dependent on ambient air during sample collection, prompting the need to consider these potential biases. Saliva and urine are far from being “ideal” samples, but our study further extended the data on the potential of untargeted metabolomics of these non-invasive samples in uncovering “unbiased” (less biased) differentiating metabolic features of allergic rhinitis and asthma in children. The identification of the metabolites contributing to the differentiating subsets of NMR spectral areas benefits future validation of the results. It may also support several pathophysiological hypotheses, namely regarding the role of arginine, taurine, kynurenine and fatty acids metabolic pathways in allergic rhinitis and asthma multimorbidity.

The work performed in this dissertation, with its strengths and limitations discussed before, achieved some contributions but generated many more questions deserving to be answered. These include several current unmet needs in asthma and its association with rhinitis, namely:

- The need to define standardized operational definitions of asthma and rhinitis to be used in epidemiologic studies. While this is not uniformly used, one possible solution is to use composite measures and report the different operational estimates, as was done in the pediatric study

estimating asthma prevalence and the elderly study estimating rhinitis prevalence. The use of such composite measures allows better comparisons with different research groups and also to estimate trends in the prevalence of the diseases and their symptoms. Nevertheless, the clinical validation of epidemiologic definitions is essential for accurate estimates. With this aim, the Control and Burden of Asthma and Rhinitis study (Impacto e Controlo da Asma e da Rinite – ICAR) combined questionnaire answers with medical doctors' evaluation and a comprehensive set of diagnostic tests, namely allergy testing together with nasal and lung function testing in a population-driven sample. This study has been designed to validate the survey instruments used in the INAsma study with clinical and objective tests and to assess and compare the burden of these diseases. We have recruited and included 263 subjects in Lisbon and Tagus Valley region in a total of 858 participants from mainland Portugal, and are currently analyzing the collected data. These should be important for the design and implementation of future studies to address asthma and rhinitis trends over the years.

- The need to define strategies for an integrated awareness of rhinitis and asthma in all age groups and increase these diseases control. Population needs should be analyzed to increase effective medical care (especially in rural areas). The ICAR study will further contribute with accurate data on the burden of these chronic diseases to set up evidence-based health policies, as it will allow to assess the effects of the diseases and their control, comparing patients with asthma or rhinitis alone, those with rhinitis and asthma multimorbidity and those without history of respiratory diseases. Several health policy strategies may be considered but they should be centered in people's needs and concerns, which are critical to an effective successful reduction in the burden of these diseases. These should simultaneously promote greater awareness and facilitate the access to accurate information on asthma, rhinitis and allergic diseases. Therefore, it is important to support patients associations and scientific societies, being closer to the general public. Strategies may take profit from the media, applications ("apps") and social networks, which are nowadays powerful tools to spread the (accurate!) word with simple and clear messages, approximating the general population to healthcare, as well as assessing population needs[41, 42, 267]. Spreading the knowledge and best medical practice on these diseases to medical students and to primary and secondary care physicians, with a strong investment in education and update, including the use of straightforward developed tools and flowcharts emphasizing patient-reported outcomes[81, 268-270], and facilitating the interaction among primary care and immunoallergy specialists can be important to reach patients at the nationwide level as well as to deliver best quality individual patient care.

- The need to further address the utility of evaluating nasal function in children. PNIF has the advantage to be cheap, simple and suitable for serial measurements of nasal function. However, more studies are needed to establish reference values and equations in pediatric age for non-decongested and decongested PNIF values. The evaluation of nasal flow using PNIF compared to other nasal function tests for diagnostic purposes and control assessment / monitoring of nasal obstruction needs to be further analyzed at the clinical level, to settle its role in every outpatient clinic that treats children with rhinitis and other causes of nasal obstruction[84, 85].

- The need to continue to pursue the characterization of asthma phenotypes and definition of endotypes. Our ongoing analyses of urine and saliva samples by gas-chromatography time-of-flight mass spectrometry are currently being undertaken in partnership with the team of experts from the Department of Chemistry of Aveiro University. This work will allow the combination of the NMR and the mass spectrometry results. Coupling these two techniques to study the metabolic profile of different samples from the same individuals greatly broadens the level of metabolite coverage that can be achieved, providing complementary information to define differentiating metabolites of childhood allergic rhinitis and asthma multimorbidity and their metabolic roles[106, 271]. Biomarker

validation is challenging but necessary in independent cohorts of biological samples, which should follow. Procedure standardization is mandatory and external factors affecting the metabolome need to be monitored. In order to allow more accurate results, samples should ideally be collected in a fasting state and the effect of drugs on the metabolome need to be carefully analyzed. Age, gender, body mass index and circadian rhythm should continue to be monitored. EBC remains an appealing matrix but potential biases, from environmental contamination to sample storage and processing, need to be carefully addressed. In particular, ambient air controls are mandatory and distinguishing exogenously originated metabolites in EBC, though challenging, is essential.

Those metabolic pathways that are identified by metabolomics approaches require further analysis to confirm their potential role in disease states. One of the pathways that has been commonly identified by different research groups as a discriminating feature in asthma profiling in different matrixes is related to purine metabolism[106, 114-117]. Apart from molecular profiling studies, adenosine has been shown to be elevated in plasma, bronchoalveolar lavage fluid and EBC of asthmatic adults compared to healthy controls[272-274]. With regard to pediatric asthma, the only published study examining samples of EBC from 11 children showed that adenosine was significantly increased in asthmatic patients[275]. Adenosine is a well-known bronchoconstrictor stimulus that is also produced endogenously by many cells during hypoxia, allergic stimulation and exercise. The net effect of adenosine depends on the relative expression of adenosine receptors on different cell types, including epithelial smooth muscle cells, neurons and leukocytes. By acting on adenosine A1 receptors, it produces bronchoconstriction and proinflammatory effects, while adenosine A2 and A3 receptors activation induces the opposite[276]. Despite the evidence regarding the role of adenosine in asthma pathophysiology, to date no selective drug directed at this molecule has proved to be safe and effective, suggesting the possibility that similar structurally or functionally associated molecules may be potential new targets or are only effective in subgroups of patients[276, 277]. Deoxyadenosine, adenosine monophosphate and inosine have been identified as significantly altered in asthma metabolomics studies[114, 115, 119]. However, to date, there are no published studies that have made the joint analysis of these adenosine-like molecules in the airways of individuals with asthma. Our research team has made several efforts to develop and validate a high performance liquid-chromatography method of increased sensitivity to quantify adenosine and related molecules and to evaluate its feasibility in EBC. Briefly, these have included derivatization coupled with fluorescence detection and a micro mass spectrometer equipped with an electrospray source and triple-quadrupole analyzer. Methodologic developments performed by the Aveiro University research team are currently ongoing to optimize the minimum volume that is needed per EBC sample for this analysis, by improving extractive efficiency and selectivity, while avoiding molecules degradation, especially during the concentration steps. Using this method, we will be able to test the hypothesis that a panel of adenosine-related molecules quantification in EBC from patients with allergic rhinitis and asthma multimorbidity differs from healthy individuals.

Asthma and rhinitis are complex diseases, where the combination of genetic information with environmental data is crucial. The combination of longitudinal raw data, especially coming from birth cohorts and moderate to severe patients' cohorts, with flexible mathematical approaches is necessary to model the effects of time-invariant and time-variant factors and increase our understanding of asthma outcomes with multiple trajectories. These should help define different therapeutic strategies for each group or even for the individual patient, to be evaluated. The addition of biomarkers (single biomarkers, in panels or composite biomarkers) is critical to predict and evaluate outcomes and treatment responses. Novel treatments should be developed together with their biomarker for the selection of responsive patients.

Multi-layered approaches that could integrate clinical data together with molecular data from genomics, transcriptomics, proteomics and metabolomics in different biological specimens provide

an opportunity to investigate the system-wide changes in asthma and rhinitis and to complement existing knowledge into mechanistic understanding[106, 107, 278]. Large scale and integrative projects adopting a systems medicine approach in asthma and other respiratory and allergic diseases are ongoing, including AirPROM, EARIP, MeDALL and U-BIOPRED. Results and achievements of these consortia are becoming available[28, 279-283], and currently leading to new disease classification hypotheses, aiming at improving the understanding of underlying pathophysiology mechanisms and better personalized disease management. The usefulness of these molecular asthma phenotypes has recently been demonstrated through transcriptomics-driven clustering approaches, namely identifying patients who may benefit from specific agents that target type 2-mediated inflammation and corticosteroid insensitivity[284, 285]. It may be necessary to use clustering methodologies that allow one element to belong to more than one cluster, instead of separate fixed individualized groups, as more than one pathophysiologic mechanism can occur simultaneously and involve common molecules. Relating molecules to their biological role will be the next step[106, 107], in order to allow a mechanisms-based approach and targeted interventions.

Big data mining may further assist to understand our current observations, in the progress from the broad asthma syndrome definition into individual "tailor-made" medicine, based in composite multiparametric profiles that may be unique to each person. This novel information can pave the way towards a new taxonomy of airway diseases[16].

Integration of artificial intelligence in clinical care will benefit building of predictive models to support decision-making and further advance into precision medicine aimed at better quality of life with more efficient, preventive strategies at the individual level. These strategies can benefit from technological and therapeutic innovations, but also from resetting current existing treatments that may have been potentially hampered by the general assumptions of current "asthma label" definition[17].

Besides the aforementioned unmet needs, others remain essential current problems in asthma and rhinitis. So far, given the chronic nature of the diseases, major issues relate to patient adherence to treatment and self-management[6, 22, 286]. Once again, approaches that consider individual needs are most likely to be successful. In particular, severe asthma continues to represent one of the most significant burdens of the disease from all perspectives of affected patients and health care system, where the definition of endotypes is urgently required for personalized treatment[287, 288]. Asthma exacerbations constitute the biggest immediate risk and anxiety to patients and their families, linked to lung function decline and huge financial burden in health care systems[2]. Preventive strategies need to be improved to avoid the effect of common triggers and other risk factors.

In summary, coupling individual unmet needs with evidence-based medicine developments will be critical for the succeeding advances in the history of rhinitis and asthma multimorbidity. Increased knowledge on pathophysiologic mechanisms opens the possibility of new efficient treatments that aim not only at the temporary relief of symptoms but to long-term disease-modifying effects. It should also contribute to global prevention strategies, answering relevant current questions at the individual level, namely: "*Will my child develop asthma/rhinitis?*", "*Is there a way to prevent these diseases development?*", "*If they develop, will it be possible to outgrow asthma/rhinitis and associated diseases?*"

The way forward is to continue to pursue the aim of understanding the beauty of health and the mechanisms involved in rhinitis and asthma multimorbidity, ultimately enabling to propose relevant therapeutic strategies for each individual person, while using our best current evidence-based knowledge and skills to prevent the disease and its effects, and promote a healthy life.

Chapter 7: References

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ATTACHMENTS – own published reviews on the topic

1. Wheezing phenotypes in childhood – is it already asthma?

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Wheezing Phenotypes in Childhood – Is it Already Asthma?

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Abstract

Wheezing in early childhood is common but highly heterogeneous. Distinguishing early wheeze phenotypes to predict long-term asthma persistence is of major clinical relevance. The comprehensive analysis of longitudinal datasets, including novel 'unbiased' statistical approaches to detect clusters from objective data, may allow better comparisons in different population settings. The recognition of such distinct outcome groups is valuable for parents' informed counselling and a prerequisite for phenotype-specific tailored interventional measures to reduce asthma burden from paediatric age until adulthood.

Keywords

Asthma, cluster, phenotype, prognosis, wheezing

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Wheezing in early childhood is among the most frequent respiratory symptoms. Despite its common occurrence, many children with early wheeze become symptom-free in later childhood and adolescence. However, early onset of recurrent wheeze is also associated with persistent asthma into adulthood, as well as to more severe, persistent lung function impairment.[1-4] This illustrates the heterogeneity of wheezing episodes in early childhood. Distinguishing different early childhood wheeze phenotypes is internationally recognised as an unmet need, since aetiology, pathophysiology, best therapy and outcome may differ.

The largest contributions to identify early childhood wheeze phenotypes came from longitudinal birth-cohort studies. The Tucson Children's Respiratory Study was one of the most relevant birth cohort studies, designed to determine wheeze risk factors.[5] A total of 1,246 new-borns were recruited and the cohort reflected a general population sample. Three phenotypes were defined: persistent wheeze (with onset <3 years of age and persisting at 6 years), late-onset wheeze (absent at the age of 3 but present at 6 years) and transient-early wheeze (onset <3 years of age but absent at 6 years). Thereafter, classifications with two to six phenotypes have been proposed, based on distinct long-term large cohort studies.[6-10] However, most of these phenotypic classifications had been limited to single disease dimensions, subjectively defined based on directly observable characteristics or *a priori* defined hypothesis. This originated distinct classifications, which are hardly comparable. Its application to different age groups or incorporation of other characteristics is restrained. Furthermore, phenotype classifications based on temporal criteria have a limited clinical use, since such groups can only be established retrospectively. In clinical practice, it may be difficult to categorise many children into mutually exclusive groups and phenotypes are not consistent over time. Currently, the relation between the different childhood wheeze phenotype definitions is not clear and no consensual classification exists.[1] Despite these difficulties, reliable phenotype definitions are important both for research and clinical practice. In this regard, wheeze phenotype definitions are valuable for informed counselling of parents and a prerequisite for the desirable phenotypic-specific or tailored treatment.

In more recent years, statistical methods that can account for multiple disease dimensions have been proposed to facilitate the unbiased identification of relevant phenotypes.[11] Such groups may not be directly observable and must be determined from objective data. Statistical methods designed to detect clusters underlying multivariable data have the advantage of avoiding the need to define phenotypes by the onset of wheeze at a given age or other pre-specified criteria. Another benefit is to simultaneously consider several disease dimensions. These methods tend to have a broader application, better allowing comparisons in different population settings. Though less biased, unsupervised cluster analysis remains dependent on the collected variables, which are still selected by the investigator. In order to improve childhood wheeze classification, the time dimension needs to be taken into account, to cope with the recognised instability of asthma characteristics in children. The wealth of data from longitudinal datasets requires the application of flexible mathematical approaches to model the effects of time-varying factors (namely environmental exposures) and wheeze outcomes with multiple trajectories, measured at different time points.

Children with poor wheezing prognosis, with regard to asthma later in life and use of preventive treatment, are mostly atopic and have personal history of rhinitis and/or eczema in pre-school age.[12-16] This supports that recurrent wheezing in early childhood associated with allergen sensitisation has a poorer prognosis, especially when

multiple early sensitisations occur. This reinforces the need to identify and quantify atopic sensitisations in early ages by *in vivo* or *in vitro* tests. Moreover, it has been shown that the reduction of exposure to multiple allergens decreased the likelihood of current asthma diagnosis by half, in children at high risk for this disease.[17] Allergen sensitisation should strengthen the diagnosis of asthma, while interventions to reduce allergen exposure may be associated with a more favourable prognosis.

Rhinitis is an independent risk factor for asthma in children and adults and it is frequently associated with atopy in children.[18,19] The association between rhinitis and asthma severity and/or control has been documented, including in children.[19] Pre-school eczema was also strongly associated with asthma persistence.[16]

Parental history of asthma in wheezing children was associated with persistent asthma, although some studies have suggested that maternal and paternal characteristics may have a distinct impact in their offspring asthma development.[20] Epigenetic changes due to environmental exposures during pregnancy or breastfeeding may account for some of these differences; exposure to tobacco smoke can also be important.

In brief, 'unbiased' phenotypic classifications derived exclusively from data are complementary to groups defined *a priori* or based on directly observable criteria. The identification and characterisation of distinct wheezing phenotypes with different prognosis, by the comprehensive analysis of longitudinal datasets will allow the institution of interventional measures to reduce the asthma burden from paediatric age till adulthood. ■

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2. Non-invasive biomarkers in asthma: promises and pitfalls

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Chapter 2

Non-invasive Biomarkers in Asthma: Promises and Pitfalls

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Abstract

The asthma concept has evolved throughout the years: one major step in asthma management is the recognition of the chronic (airway) inflammation; another major step is further understanding of asthma heterogeneity and subsequent development of targeted therapies. While the concept of chronic inflammation, airway structural changes and their variability over time are widely accepted, their measurement and monitoring have gone through many hardships.

In this chapter, we discuss the need for applicable biomarkers in asthma management and focus on the currently available and most promising totally non-invasive samplings and detection techniques, ranging from single biomarkers to biomarker panels and composite signatures, including molecular high-throughput "omics" technologies outcomes. Limitations of these biomarkers are compared with minimal-, semi- and invasive techniques. Additionally, we discuss the benefits of an integrative systems medicine approach, considering asthma phenotypes based on cluster analysis of multidimensional biomarker datasets and its contribution to recent developments towards the promise of better understanding asthma and personalised asthma management.

Keywords: asthma, biomarkers, composite signature, phenotype, personalised medicine

1. Introduction

According to the concurrent paradigm, asthma should not be regarded as a single disease, but rather as a complex of multiple, overlapping syndromes. The heterogeneity of asthma has been

recognised already for over a century, for instance, as intrinsic and extrinsic ("allergic") asthma [1].

The introduction and subsequent validation of hypertonic saline-induced sputum analysis revealed different inflammatory asthma phenotypes: i.e. eosinophilic versus non-eosinophilic [2]. Asthma phenotypes comprise shared similar observable characteristics, produced by the interactions of an individual's genetic make-up and the environment that can be affected by several triggers and respond to treatment. However, phenotypes may vary over time and do not directly link to the underlying pathophysiology. Factor analyses involving various disease characteristics and biomarkers, including fractional exhaled nitric oxide (FeNO) levels and sputum cell differentials, helped to further define asthma (sub)phenotypes [3, 4].

In the 1990s, in analogy with animal models, asthma was thought to be a typical T-helper (Th)2- and immunoglobulin E (IgE)-driven disease, and hence, the proof of clinical effectiveness of potential asthma therapeutics was tested in the allergen challenge model. More recently, genomics and other sophisticated "omics" techniques enabled further characterisation of various inflammatory cells and other biomarkers, and helped to link asthma subphenotypes or endotypes to specific cellular and molecular pathways. For instance, gene expression profiling revealed two major subtypes: i.e. "Th2-high" and "Th2-low" asthma providing evidence for responders and non-responders to Th2-targeted therapies [5, 6]. Apart from the involvement of the adaptive immune responses, pathognomonic for parasites and allergens, more recent insight showed the major involvement of the innate system (ILC2s: innate lymphoid type 2 cells) in some asthma endotypes [7]. Interestingly, both Th2 cells and ILC2s produce type 2 cytokines (i.e. interleukin (IL)-4, IL-5 and IL-13) and these type 2 responses are mainly mediated by eosinophils. However, the underlying "upstream" mechanisms differ: while allergens mainly drive Th2-responses [8], viruses and pollutants are common triggers for ILC2-mediated type 2 responses that involve epithelial cells and IL-25, IL-33 and thymic stromal lymphopoietin [9]. Presently, it is not fully clarified how exactly both type 2 response pathways interrelate.

Apart from disease typing, the discovery of new inflammatory pathways and related biomarkers resulted into the development of endotype-specific, individualised asthma treatment.

In this review, we aim to highlight the key non-invasive and semi-invasive biomarkers currently used in the management of asthma.

2. Do we need biomarkers in asthma?

Given the heterogeneity of asthma and the evidence that standard therapy is not (fully) effective in all patients, especially in those with more severe disease and those at risk for frequent exacerbations, the need for appropriate biomarkers allowing the identification and subsequent targeted treatment of these patients has been increasingly recognised. Since asthma is multidimensional and thus presents at several different levels including clinical, physio-

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logical, histological, cytological and molecular, various approaches have been developed to identify effective biomarkers (**Table 1**) [10]. In addition, given the complexity of the disease, (unbiased) biomarker clustering within different asthma populations has been performed by several research groups, which revealed different disease subphenotypes with varying disease course and/or response to treatment [3, 4].

Disease level	Parameters/biomarkers
Clinical	Age of onset Frequent exacerbators Therapy resistance Cofactors, including allergy, nasal polyps, recurrent viral infections, air pollutants including passive and/or active tobacco smoke, obesity
Physiological	Lung function (normal, reversible, fixed obstruction) Airway hyperresponsiveness
Cytological	Inflammatory cells and soluble markers in: Sputum (central airways); BAL, bronchial wash/brushings (peripheral airways)
Histological	(Trans)bronchial biopsies (inflammatory and structural cells and structures)
Exhaled air	FeNO (fractional exhaled nitric oxide) EBC (exhaled breath condensate) VOCs (volatile organic compounds: eNose) EBT (exhaled breath temperature)
Systemic biomarkers	Peripheral blood: eosinophils, CRP, IgE, periostin, cytokines
Molecular	Genomic SNP analysis (i.e. the large-scale genotyping of single nucleotide polymorphisms) Transcriptomic analysis (i.e. the measurement of all gene expression values in a cell or tissue type simultaneously) Proteomic analysis (i.e. the identification of all proteins present in a cell or tissue type) Metabolomic analysis (i.e. the identification and quantification of all metabolites present in a cell or tissue type; eNose)

BAL: bronchoalveolar lavage; CRP: C-reactive protein; eNose: electronic nose; IgE: immunoglobulin E; SNP: single nucleotide polymorphism

Table 1. Clinical and biological biomarkers in asthma.

Using a systems biology approach in large cohorts of patients, researchers within the Innovative Medicines Initiative Severe Asthma Project U-Biopred have been collecting data, including molecular analyses, tissue, exhaled air and blood samplings, as well as clinical and lung function data, and patient-reported symptoms [11]. By combining this information, the researchers aimed to generate a "handprint", i.e. a combination of clinical and biological

characteristics (biomarkers) indicative of a specific asthma subphenotype/endotype. Subsequent studies are being undertaken to test if one's "handprint" can predict the disease course and can indicate a response to (targeted) asthma treatments. This approach will provide a key step to personalised medicine [12–14].

Generally, an ideal biomarker should possess the following key characteristics: clinical relevance, adequate sensitivity and specificity for (targeted) treatment effects, repeatability, simplicity and cost-effectiveness [10].

3. Promising single non-invasive biomarkers of asthma

The concept of asthma has undergone considerable changes throughout the years, from a disease mainly manifesting by variable symptoms and bronchoconstriction to airway inflammation and remodeling. More recently, heterogeneity has gained an outstanding position in asthma definition. So far, one of the most important steps in asthma history, bringing significant reduction in morbidity and mortality, was the recognition of airway inflammation in asthma and the introduction of efficacious and safe anti-inflammatory therapy for asthma control. Despite ongoing developments, current guidelines for both diagnosis and follow-up of patients with asthma are still grounded on clinical and lung function parameters. Thus, functional biomarkers were the first objective measures coming forward into clinical practice and, in general, the promise of delivering valuable molecular, cellular or histological biomarkers to daily clinical practice has not yet been met. However, intense research in asthma has brought together scientists from academia, research institutes, the pharmaceutical industry and patient organisations, with significant progress taking place in the recent years. In this section, we discuss the currently available and more advanced non-invasive biomarkers in asthma.

Clinicians and researchers dedicated to asthma may benefit from a direct analysis of the airways, profiting the patients. In fact, non-invasive airway assessment is possible through lung function tests (LFTs) and airway sampling. Furthermore, other "more distant" to the airway biomarkers (such as blood or urinary biomarkers) can also be regarded as potentially useful, considering the systemic properties of asthma.

3.1. Functional biomarkers

LFTs are essential in routine clinical practice. They are non-invasive, well validated and reproducible. At present, LFTs provide the only generally accepted functional biomarkers to objectively aid in the diagnosis, risk assessment and monitoring of asthma. Thus, asthma definition currently implies the objective detection of variable airflow limitation, while the "best personal lung function" is a hallmark of asthma monitoring and future risk assessment.

LFTs provide relative features (phenotypes) that aid in differential diagnosis, namely in the distinction from chronic obstructive pulmonary disease (COPD), but are not diagnostic in its use. For instance, neither post-bronchodilator airway obstruction, lack of bronchodilation response or hyperinflation can be used to rule out asthma.

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Presently, LFTs patterns alone are not considered to define disease subsets that respond to particular therapies. However, lung function has been shown to be predictive of clinical outcomes and provide complementary information to subphenotype asthma. For instance, variability measures of lung function can predict the loss of asthma control and response to long-term beta2-agonist treatment [15].

Airway hyperreactivity (AHR) is a basic pathophysiological hallmark of asthma, but remains a complex component of this disease. A growing number of variable airway smooth muscle (ASM) and non-muscle factors contributing to AHR has been recognised. Besides its high negative predictive value in the diagnosis of asthma, AHR has been advocated as a surrogate biomarker related to airway inflammation to guide asthma management. It has been shown that anti-inflammatory therapy directed at reducing AHR may imply higher corticosteroid doses, but leads to improved lung function and better control [16, 17]. AHR evaluation has also been suggested useful in back titration of inhaled corticosteroids. However, the reduction in AHR with higher doses appears targeted to the persistent structural component of AHR (defined as opposed to the variable inflammation component of AHR). Emerging data support that it is the structural changes of the airway that mainly contribute to AHR (i.e. reticular layer thickness and ASM hypertrophy) [16]. This effect also depends on the type of challenge used: assessing AHR to indirect bronchoconstrictor stimuli is superior in the detection of changes associated with airway inflammation, while direct stimuli, mediated through direct interaction with ASM, better reflect the structural changes. Assessment of AHR is a useful non-invasive tool providing complementary information, though its routine feasibility in general practice can be hard to settle.

Summing up, lung function measurements may not, per se, reflect the precise underlying pathological processes responsible for different phenotypes. However, in a multidimensional approach to evaluate asthma as a complex dynamic disease, functional biomarkers and their variability must definitely be part of future composite parameters in asthma.

3.2. Exhaled air biomarkers

Exhaled breath can be sampled in a fully non-invasive manner across all age groups. However, exhaled breath analysis is not useful for analysing cellular or histological biomarkers and, in general, the search for useful molecular biomarkers has been hampered by methodologic difficulties mainly dealing with very low molecular concentrations, variability and lack of sampling and analysing methods standardisation [10].

FeNO is so far the most commonly used molecular biomarker in exhaled air. Nitric oxide (NO) is a gaseous chemical compound, which can be measured in exhaled breath either by chemiluminescence and electrochemical analysers. The American Thoracic Society and the European Respiratory Society recommendations for standardised procedures for the FeNO measurement have been published [18]. Accordingly, FeNO is measured at a flow rate of 50 mL/s, thus reflecting NO production from the central airways. Currently available devices allow accurate and highly reproducible measurements, through simple, fast and non-invasive methodology. Hand-held devices are now widely available in clinical practice and used in both adults and children (since preschool age, usually above the age of 4 years) [10].

Evidence-based guidelines for adequate interpretation of FeNO measurement have been developed [19]. This biomarker can be affected by several perturbing factors, mainly age, height and recent active or passive smoking. Other variables that have been reported to affect FeNO levels include weight, gender, race, atopic status, diet or alcohol intake [20]. Large variation of normal FeNO values exists, with wide inter-individual differences and significant overlaps between healthy/non-asthmatic and asthmatic populations. Intriguingly, the aforementioned confounding factors explain few of the substantial variations within the general population [20]. For these reasons, guideline-recommended cut-points are supported for routine interpretation of FeNO levels [19].

Presently, there is evidence to support the use of FeNO thresholds essentially for assessing the likelihood of Th2-mediated airway inflammation and responsiveness to corticosteroids [19]. Low FeNO levels do not rule out asthma [19].

Persistently high FeNO levels may be attributed to poor adherence to corticosteroid therapy, poor inhaled drug delivery or persistent/high allergen exposure [19]. This has also been suggested to reflect a highly reactive asthma phenotype [21]. Although FeNO may be indicative of loss of disease control or exacerbation, some patients remain with high FeNO despite good clinical asthma control, and clinical trials of FeNO-guided management have yielded conflicting results [22–24]. Increased knowledge on asthma pathophysiology and the source and biochemistry of FeNO may help to further understand these findings. Traditionally, FeNO is known to originate in the airway epithelium as a result of inducible nitric oxide synthase (iNOS) upregulation, which occurs with inflammation [19]. Recent data give further support to this view by showing iNOS overexpression in the airway epithelium of patients with asthma [25]. However, it is interesting to note that despite the strong association between FeNO and Th2-mediated/eosinophilic inflammation and atopy, eosinophils are not the principal cells in the airways that express iNOS and this enzyme is upregulated by Th1 cytokines [26]. Anti-IL-5 and anti-IgE therapy for asthma reduced sputum eosinophilia without affecting FeNO, contrary to IL-13 inhibition that significantly decreased FeNO [27]. Studies have shown that FeNO levels are not elevated in many patients with severe asthma, compared to mild and moderate asthma, despite evidence of airway inflammation [13, 28]. Other sources of FeNO need also to be considered. For instance, as NO is a highly reactive molecule, it can be trapped and directly regenerated by abundant free thiol-containing biomolecules [26]. One of these thiols is S-nitrosoglutathione, which has been shown to be depleted in severe asthma, possibly contributing to comparative lower FeNO levels in these patients. Another important reservoir of nitrogen species is nitrite/nitrous acid. These agents are physiologically recycled in blood and tissues to form NO and other bioactive nitrogen oxides. When airway pH increases, more nitrite is formed and FeNO levels fall. On the other hand, FeNO may be high with acidification [26]. Still, many questions regarding the source of FeNO and its specific role need to be explored. Another area of research that may bring additional knowledge and clinical usefulness is dedicated to partitioning of FeNO. In particular, alveolar FeNO can be obtained by measuring FeNO at multiple flow rates and has been shown to be an independent parameter that is putatively associated with increased distal lung inflammation and more severe disease [29].

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In summary, the clinical importance of FeNO as a marker of Th2-mediated airway inflammation that is likely to respond to corticosteroid treatment may be "indirect," but is well established. Further analysis is needed to address the possible need to define FeNO levels cut-points in different situations, according to the presence or absence of pertinent confounders. The application of FeNO measurement to identify particular asthma phenotypes or as part of a more comprehensive panel of biomarkers including also other "Th2 type" biomarkers may allow taking better profit of this readily available biomarker [30]. Partitioning of FeNO is a promising area of research, whose clinical usefulness is yet to be established.

Other biomarkers have been studied in exhaled breath vapor namely *volatile organic compounds* (VOCs). In general, reactive oxygen species result from inflammation and promote polyunsaturated fatty acids degradation, originating volatile hydrocarbons. These VOCs are subsequently excreted in exhaled breath. Thus, exhaled VOCs may originate from systemic metabolism or from local airway inflammation. It is important to consider also that VOCs in exhaled breath may also be originated from pathogenic bacteria or from exogenous sources such as ambient air pollution [31]. Some studies have suggested that single VOCs such as pentane or ethane could be significantly higher in patients with asthma. However, VOCs profiles analyses bring significant additional value [31].

Another potential single biomarker in exhaled air is *exhaled breath temperature* (EBT), which reflects heat, a cardinal sign of inflammation. EBT has been shown to correlate with bronchial blood flow [32], which is advocated as the main mechanism to explain EBT changes in disease status.

Several studies have shown that EBT is higher in patients with asthma [32–34]. Conflicting data have been reported regarding a possible association between EBT and asthma control, with several studies supporting [34, 35], and others rejecting this relation [36, 37]. Correlation between EBT and other biomarkers, such as sputum eosinophils and FeNO, has resulted in inconsistent reports [32, 37]. Furthermore, EBT has been shown to increase after eucapnic voluntary hyperventilation, methacholine challenge test or exercise, but no difference was found between asthmatics and healthy individuals [38], suggesting this increase in EBT to be physiologic.

However, it is important to stress that different methods have been used to measure EBT. Some studies used a flow and pressure-controlled maximal slow continuous exhalation to residual volume to measure EBT, while others measured EBT in tidal volume until a temperature plateau was reached. Different variables have been analysed: plateau EBT, rate of temperature increase, time to achieve plateau EBT. These different methods preclude results comparison and, to our knowledge, no study has analysed both methods simultaneously. The recent development of improved, easier-to-use, portable devices has improved feasibility, including in children and in the elderly [34, 36, 39].

Moreover, further studies are needed when it concerns interpretation of the results. Variables such as room temperature and relative-ambient humidity may influence the results [39]. Some studies point a correlation between gender [37, 39], age [36, 39] and lung volume [36], which

needs to be addressed. No significant correlation has been documented between EBT and auricular temperature, suggesting EBT to be a distinct variable and not just another measurement of body temperature [33, 34].

Conclusively, EBT assessment may be an appealing method enabling completely non-invasive and patient-friendly evaluation and deserves further standardisation and validation as a potentially useful biomarker in asthma.

3.3. Exhaled breath condensate biomarkers

Exhaled breath has been a source for intense research in the latest years and many other biomarkers have been studied. *Exhaled breath condensate* (EBC) has the advantage of being a more stable matrix than exhaled breath vapor, including volatile and also non-volatile compounds. It is obtained by cooling exhaled air and is thought to reflect the composition of the airway lining fluid. Many molecules have been analysed in EBC, including metabolites and also proteins. Although methodological recommendations for exhaled breath sampling and analysis have been published [40], the procedures for EBC collection and biomarker detection are not fully standardised and there is significant heterogeneity between different working groups yielding (highly) variable data.

Many biomarkers analysed in EBC reflect oxidative stress. Among these, the most extensively studied include H_2O_2 and isoprostanes.

H_2O_2 is a reactive oxygen species that contributes to oxidative stress within the airways. A meta-analysis has reported that EBC H_2O_2 concentrations were significantly higher in adults with asthma, and associated with disease severity and control [41]. This has also been reported in children. Of importance, smoking increases H_2O_2 levels. EBC H_2O_2 levels were inversely correlated with lung function parameters and improved with inhaled corticosteroids [41]. Thus, EBC H_2O_2 has been suggested a promising biomarker for asthma control monitoring.

Oxidative stress can also be assessed through the determination of lipid peroxidation-derived products. *8-isoprostane* derives from arachidonic acid peroxidation. Increased levels of 8-isoprostane have been found in EBC in patients with asthma, correlating with disease severity [42]. EBC 8-isoprostane levels have been shown to be particularly useful to indicate asthma control and severity in childhood when combined with different markers [30]. Increased 8-isoprostane levels in EBC of children with exercise-induced bronchoconstriction (EIB) have been described, suggesting a role for oxidative stress in EIB [43].

Markers of inflammation have also been addressed. *Leukotrienes (LT)* are important mediators of airway inflammation in asthma, and the most extensively studied molecular biomarkers of inflammation in EBC. Increased levels of LTs have been detected in EBC of patients with asthma, correlated with disease severity and were effectively reduced by oral corticosteroids or LT receptor antagonist [44, 45]. However, the reported effect of inhaled corticosteroids on LTB4 EBC levels is controversial [46]. LTs have been suggested as markers of asthma severity [42]. Likewise, LTs have been associated with EIB severity [47].

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Various *cytokines* and other molecules have been analysed in EBC. In particular, IL-4 has been found to be higher in EBC of patients with asthma, especially in asthma associated with atopy [30, 42]. Cytokine ratios and biomarker panels in EBC including cytokines have been suggested to be useful to assess asthma control (including IL-4 and interferon-gamma) and to predict asthma exacerbations (e.g. IL-5) [30, 48].

Last but definitely not least, the measurement of *pH* is one of the simplest and most technically validated biomarkers in EBC. EBC pH reflects airway acidification [49]. Several research groups have found higher pH levels in healthy subjects, compared to patients with asthma [10]. Significant decline in EBC pH occurred during asthma exacerbations. EBC pH shows good reproducibility, having low running costs and normal data sets have been published in self-reported healthy subjects [50].

Although some biomarkers may be useful to measure in EBC, samples are highly diluted, biomarker concentrations are difficult to measure, require specialised equipment, laboratory techniques and normalisation standards are lacking. Unfortunately, EBC has been hampered by serious drawbacks in the methodology, detection techniques and result interpretation, all consistent with large intra and intersubject variability, precluding validation for most single biomarkers.

3.4. Biomarkers in non-respiratory specimens

Other non-invasive matrices have also been analysed in search for biomarkers in asthma. *Saliva* is a readily available specimen and allows metabolites, proteins and also deoxyribonucleic acid (DNA)/ribonucleic acid (RNA) extracting (although buccal swabs perform better), including also oral microbiota assessment. *Cotinine* in saliva has been one of the most extensively studied biomarkers, with interest in asthma as a measure of tobacco exposure. Salivary *cortisol* has also been used for the evaluation of adrenal function. Morning salivary cortisol was significantly lower in patients with asthma than in healthy individuals, and poor asthma control has recently been associated with lower salivary cortisol levels [51]. Preliminary data have suggested that *inflammatory salivary markers* may also be associated with asthma control, including eosinophil-related (such as eosinophil cationic protein) and myeloid/innate mediators [52]. Additionally, a significant decrease in salivary antioxidant enzyme-peroxidase activity was observed in children during asthma exacerbations [53]. A salivary pH decline has also been associated with asthma and AHR [54]. Another area of research includes the analysis of oral microbiota, which may change in asthma, either through disease status or its pharmacotherapy. The interest in saliva studies in relation to asthma is still preliminary and the role of many possible confounders needs to be considered.

Although *urine* does not directly reflect the airways, samples are easily obtained across the full age spectrum. Several urine molecular biomarkers have been described to be associated with asthma. Here, we focus on four molecules which have been studied in more detail.

Of the potent lipid inflammatory mediators comprising the cysteinyl *LTs*, only LTE4 is stable, making this molecule the dominant LT detectable in biological fluids. Urinary levels of this end product of LT metabolism have been shown to be elevated in asthma, both in children and

adults, and in patients with aspirin-exacerbated respiratory disease [55, 56]. It has been associated with the degree of airflow limitation and acute exacerbations [55, 57]. Although inhaled corticosteroids are the most effective treatment for asthma, they do not alter LTE4 excretion. Urinary LTE4 levels have been suggested as potential predictors of better response to anti-LT therapy compared to other therapeutic approaches, though further studies are needed, including other biomarkers, to predict individual responses.

As LTs, *prostaglandins* (PG) are the end products of arachidonic metabolism. PGD2 results from cyclooxygenase pathway and is excreted in urine after being metabolised to 9α,11β-PGF2. Increased urinary excretion occurs in patients with asthma and after challenge tests, and a negative association has been found with lung function [58].

Bromotyrosine is another molecule with possible interest in asthma. It is generated from protein oxidation by eosinophils. The oxidised amino acid is stable and excreted in urine. Urinary bromotyrosine levels are higher in patients with asthma and have been associated with asthma control and lung function, predicting exacerbations [59]. Its levels have been shown to reduce during inhaled corticosteroid therapy. High urinary bromotyrosine levels could predict a favorable clinical response to inhaled corticosteroid therapy, especially in combination with high FeNO values [59]. These results warrant further developments.

Though urinary biomarkers may become useful tools, many require specialised equipment and their measurement is not fully validated or standardised. There is a current need for normalisation standards and assessment of intra and inter-individual variation to select the potentially useful biomarkers. It is also important to address urine dilution when reporting quantitative absolute results.

3.5. Airway imaging biomarkers

Airway imaging biomarkers are also emerging, offering the potential of adding complementary information, namely on small airways function and remodeling. High-resolution computed tomography (HRCT) images are used to measure airway narrowing, wall thickening, air trapping and ventilation inhomogeneity [27]. The first two measures have been correlated with lung function and asthma severity. Increased parenchymal lucency has also been associated with severe asthma exacerbations, lung function and neutrophilic inflammation. HRCT is easily performed though it requires that lungs are scanned at a standard volume for validity and reproducibility. The risk of exposure to significant ionising radiation needs to be considered and normal ranges have not been established.

4. Composite biomarkers in non-invasive sampling: what is known and what could be useful in the future?

The complexity and dynamics of asthma drive the need to establish distinct disease phenotypes and endotypes. There are several different triggers in asthma, with various pathophysiological pathways in parallel resulting in clinical expression that may be rather similar. Therefore,

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repeated multiscale, multidimensional measurements may be needed to capture this complexity, which may yield more useful information than single or even panels of combined biomarkers [60]. In this view, molecular composite signatures may be obtained by high-throughput "omics" technologies, which are increasingly standardised. Several large-scale studies of the genome, transcriptome, proteome or metabolome have produced an enormous amount of data and it is pivotal to follow the available guidelines in order to avoid false discoveries [60]. The composite high-dimensional signatures or fingerprints are based on pattern recognition underlying complex non-linear biology systems. Some evidence that this approach may be successful in asthma has already emerged concerning differential diagnosis [61]. Regarding non-invasive, direct assessment of exhaled breath, it is interesting to note that while many problems arise in specific molecular biomarkers validation, recent studies have shown encouraging results with the application of metabolomics strategies to study exhaled biomarkers [60, 62, 63].

Among "omics" systems biology, metabolomics is considered the one that comes closest to phenotype expression. It involves the identification and quantification of small molecular weight metabolites. Real-time metabolomics measurements are already feasible for several clinical applications with electronic noses (eNoses). These handheld, portable devices can capture various combinations of VOCs in exhaled breath, with nanosensors arrays. The nanosensors are based on conducting polymers, metal oxide, metal oxide field effect transistors, surface or bulk acoustic waves, optical sensors, colorimetric sensors, ion mobility spectrometry, infrared spectroscopy, gold nanoparticles and gas-chromatography (GC) coupled with mass spectrometry (MS) or flame ionisation detection [60, 64]. The pattern recognition algorithms using various eNose sensor systems indicate fingerprints of exhaled VOCs, called breathprints, which have shown to discriminate patients with asthma from healthy subjects and COPD with accuracies between 80% and 100% [65]. Breathprints have also been studied to phenotype asthma. Recent studies indicate that eosinophilic and non-eosinophilic asthma can be distinguished when using a composite eNose platform. Breath analysis by eNose could also predict the response to corticosteroids with greater accuracy than sputum eosinophils or FeNO [66]. These data suggest that composite signatures of breath analysis could be used for assessment and monitoring of airway inflammation. Important methodologic issues of technique optimisation and standardisation deserve deeper analysis, from breath sampling, to modulating factors including comorbidities and incompatibility between eNoses. These should enable external validation to determine possible disease-specific breathprints with clinical applicability.

Besides the analysis of exhaled breath vapour with GC-MS and eNoses, the novel metabolomics approach has also been applied to EBC. It has been shown to enable characterisation of metabolic compounds in even small EBC volumes, using high-resolution proton nuclear magnetic resonance (NMR) or MS. This has proved capable of discriminating healthy individuals from those with asthma [62, 63, 67, 68]. It could also discriminate between severe and non-severe asthma [63], supporting the hypothesis that severe asthma has specific metabolic features.

Interestingly, the metabolomics analysis of urine also discriminated healthy individuals from those with asthma [69], and could distinguish patients with stable asthma from those with acute exacerbations based on profiles [69, 70]. Metabolomics analysis of urine samples has also been recently suggested as a useful clinical tool to differentiate asthma from COPD [71].

Pinkerton et al. [72] demonstrated for the first time that differences between healthy controls and asthma patients could be detected via micro-RNA (miRNA) expression in EBC, and suggest that different types of inflammation may have unique miRNA signatures. These small non-coding RNAs are known to be important in the post-transcriptional regulation of inflammation, thus opening a new research field using non-invasive direct air sampling.

Proteomics has also recently been applied to EBC. Liquid chromatography (LC)-MS has been used to separate and detect proteolytic peptides present in EBC with differentiating profiles based on asthma status [73]. However, this preliminary study faced several problems such as insufficient sample volume, possible salivary contamination and difficulties in peptides identification due to their low concentration.

Besides allowing an overview of molecular signatures, the "omics" approach may potentially lead to new knowledge regarding asthma pathophysiology, due to its untargeted, hypothesis-generating approach. All biomedical researchers are facing not only the opportunities but also the challenges in accessing, managing, analysing and integrating diverse data sets that are larger, more diverse and more complex than ever before, and that exceed the abilities of current management and analysis approaches [60, 74]. Composite biomarkers research such as that coming from molecular profiling assays including various "omics" is a live example that needs to be critically interpreted and cautiously validated to yield truly significant advances in personalised medicine.

5. Non-invasive biomarkers limitations: can more invasive sampling do better?

Asthma syndromes are characterised for being dynamic, with varying changes in symptoms pattern, lung function, inflammation and remodelling throughout time. In this setting, non-invasive direct airway sampling, such as exhaled breath analysis, seems especially appealing, allowing easy and repeatable measures over time. However, low molecular concentrations and variable sample dilution lead to difficulties in methods sensitivity and validation, with consequent issues in replication of biomarker findings (**Table 2**). In comparison, bronchoscopy allows direct visual examination of the airways and direct collection of fluid (bronchoalveolar lavage, bronchial washing) and tissue (brushing, biopsy). These techniques are mostly impractical because they are invasive, require specialised equipment and qualified personnel, have contraindications and carry potential risks / complications. Therefore, ethical issues preclude bronchoscopic sampling broad use in asthma, even less when repeated samplings are needed, thus being mainly reserved for selected severe patients and for research purposes. Apart from practical issues, standard bronchoscopy techniques hold several other limitations, including lack of reproducibility and sample dilution effect, despite recently proposed

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improvements (Table 2) [75]. In between invasive and non-invasive airway samplings, semi-invasive induced-sputum analysis may also reflect the airways and is easier to perform. Moreover, although indirect, blood sampling is minimally invasive and is a known relevant biomarker source in asthma.

Biomarker source	Pros	Cons
Exhaled breath	Totally non-invasive Validated for FeNO measurement Portable (FeNO, eNose, EBT, EBC) Direct results (FeNO, eNose, EBT) Multiple molecular biomarkers May be collected across all ages May be collected in severe patients Allows serial measurements	Validation not complete (except FeNO) Many perturbing factors Upper airways/salivary possible contamination Require expertise, expensive and time-consuming specialised lab assays (EBC) Soluble markers subject to dilution
Induced-sputum	Semi-invasive Validated tool Molecular and cellular biomarkers Useful to guide treatments (sputum-eosinophils)	Impossible in young children Contraindicated in severe bronchoconstriction / active cardiovascular disorders Rescue medication / procedures needed Non-repeatable over short time-period (<12 to 18 h) Procedure itself may induce changes in airways/lab results Upper airways/salivary possible contamination Require expertise, expensive and time-consuming specialised lab assays Soluble markers subject to dilution
Bronchoscopy	Direct airway assessment	Invasive Several medical contraindications Rescue medication/procedures needed Non-repeatable in many patients Expertise and experience required for procedure Require expertise, expensive and time-consuming specialised lab assays BAL markers subject to dilution Procedure itself may induce changes in airways/lab results (BAL)
Blood	Minimally invasive Some biomarkers routinely available (e.g. eosinophil counts) Useful to guide treatments (e.g. eosinophils counts) Molecular and cellular biomarkers May be collected across all ages May be collected in severe patients Allows serial measurements	Not directly reflecting the airways Not patient-friendly in all subjects (e.g. children) Require expertise, expensive and time-consuming specialised lab assays (some biomarkers)
Urine	Totally non-invasive May be collected across all ages May be collected in severe patients Allows serial measurements	Not directly reflecting the airways Require expertise, expensive and time-consuming specialised lab assays

BAL: bronchoalveolar lavage; EBC: exhaled breath condensate; EBT: exhaled breath temperature; eNose: electronic nose; FeNO: fractional exhaled nitric oxide.

Table 2. Pros and cons of main biomarker sample sources in asthma.

In this section, we will discuss these sampling methods and related current main biomarkers for asthma management.

5.1. Sputum biomarkers

Induced sputum is a validated sampling method of the more central airways. Sputum is collected after inhalations of hypertonic saline. Although relatively safe, induced-sputum requires specialised training, equipment and laboratory processing. Monitoring lung function during the induction procedure reduces the risk of excessive bronchoconstriction. Patient's active cooperation is needed for collection, making this technique unsuitable for some patients, especially for children below the age of 7 years [76].

Induced-sputum provides a rich source of soluble and cellular biomarkers and has exceptionally allowed a successful single biomarker-based clinical management approach in asthma. This is the case with sputum eosinophil percentage, which identifies patients who have eosinophilic and non-eosinophilic asthma phenotypes and can be predictive of poor asthma outcome and targeted treatment response, with demonstrated treatment-guided superior efficacy in reducing asthma exacerbations in adults [2, 27, 77, 78]. Thus, sputum eosinophil percentage acts as a key marker and correlates with severe exacerbations and AHR. It has also been useful in a panel of biomarkers to select patients who may benefit from IL-5 targeted therapies, including mepolizumab (anti-IL-5), reslizumab (anti-IL-5) and benralizumab (anti-IL-5R). In contrast with adults [77, 78], eosinophil sputum-guided therapy was not associated with decreased asthma exacerbations or improved asthma control in school-aged children and adolescents [79]. Sputum inflammatory phenotype was shown to be unstable in children with asthma, and this was not related to treatment or disease control [80].

Besides eosinophils, other sputum biomarkers are currently in research. Sputum neutrophils are often related to severe non-eosinophilic asthma with fixed airway obstruction. Soluble sputum biomarkers have been associated with asthma severity (e.g. eosinophilic cationic protein, LT, IL-4, IL-5, IL-13, IL-6, IL-12, tumour necrosis factor-α, granulocyte-macrophage colony-stimulating factor), exacerbations (e.g. IL-8, neurokinin A) or remodelling (procollagen synthesis peptides, tissue inhibitors of metalloproteinase or transforming growth factor-β) [10]. Many biomarkers can be measured, but most require highly sensitive detection methods and results may be affected by sputum processing or variable dilutions. These factors need to be taken into account to select and validate useful biomarkers in sputum.

Induced sputum may also be an interesting source for composite biomarkers. Unsupervised clustering of induced-sputum gene expression profiles identified three transcriptional asthma phenotypes that related to clinical and inflammatory parameters (resembling eosinophilic, neutrophilic and paucigranulocytic asthma) [81]. Differentially expressed genes were related

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to immune and inflammatory responses, proving a framework to investigate asthma endotypes.

In summary, logistic and practical difficulties have precluded the wide use of induced sputum in clinical practice, but sputum eosinophil percentage is recommended as a supplemental measure in future asthma clinical research studies to identify specific cellular profiles and to predict or to monitor a treatment response in adult patients [27]. It is important to highlight that sputum eosinophils and FeNO are not duplicative outcome measures, even though low sputum eosinophil and low FeNO are strongly linked [27].

5.2. Blood biomarkers

Peripheral blood can be collected across all age groups, with minimal risk. Some biomarkers are routinely standardised in medical institutions and therefore readily available, such as eosinophils, total serum IgE and allergen-specific IgE. The latter are used to define atopy, which can be accurately, easily and more readily detected by skin prick test. Atopy modestly increases the probability of asthma, but is not essential for diagnosis. Though it is useful to characterise patients, atopy itself is recognised to be heterogeneous, including both "Th2-high" and "Th2-low" phenotypes [5]. Specific sensitisations are useful in clinical practice to suggest clinically relevant allergen avoidance and consider allergen-specific immunotherapy. However, total IgE or allergen-specific IgE quantification cannot predict the response to treatment and are otherwise weak biomarkers in asthma.

Blood eosinophil absolute count has long been associated with asthma and remains a recommended supplemental asthma biomarker [27]. Although it may not reflect the airways and be unspecific, blood eosinophilia supports asthma diagnosis and is an independent risk factor for exacerbations and fixed airflow limitation. Blood eosinophil counts are useful to subphenotype asthma and to monitor systemic biologic effects of pharmacologic interventions in patients with asthma, including (inhaled) corticosteroids, anti-IgE, LT antagonists and 5-lipoxygenase inhibitors [27]. Furthermore, blood eosinophil counts emerged as predictive biomarkers of clinical benefit from IL-5- and IL-13-targeted therapies, being associated with a "Th2 bronchial signature" [82].

Another promising "Th2-high" serum biomarker is the extracellular matrix protein periostin. The expression of periostin is upregulated by IL-13 in bronchial epithelial cells and, unlike IL-13, is abundant and readily detectable in peripheral blood [82]. Interestingly, a multi-centre study collecting matched sputum, bronchoscopy and peripheral blood samples from patients with asthma showed that serum periostin was the best single predictor of airway eosinophilia, with a further advantage of lower intrasubject variability over time than FeNO or blood eosinophilia [82]. However, conflicting results have recently been reported [83, 84]. Nevertheless, periostin levels have been associated with asthma severity and its levels have also been shown to be important to predict lebrikizumab (anti-IL-13) clinical benefit, with greater reduction in severe exacerbations and greater improvement in lung function in the "periostin-high" patients [85]. A greater decrease in exacerbations with anti-IgE therapy has also been reported in "periostin-high" patients. Healthy subjects and lebrikizumab-treated patients still

have measurable levels of serum periostin, thus other systemic sources of periostin than IL-13 need to be explored [82].

Overall, blood eosinophils, serum periostin and FeNO reflect "type 2" airway inflammation in different ways and are only weakly correlated; therefore, combinations of these biomarkers obtained with minimally or non-invasive samplings may further enable optimisation of treatment benefit [82, 86, 87].

Recently, application of "omics" technologies to peripheral blood and invasive sampling with unsupervised clustering are yielding crucial data to capture the complexity of various asthma phenotypes and add new insights on asthma endotypes and treatment response. Given its maturity, transcriptomics analysis using microarrays is the current state-of-the-art method for asthma signature discovery [60]. For instance, gene expression profiling of bronchial epithelium identified distinct subtypes of patients with asthma with "Th2-high" or "Th2-low" phenotype [5], supported the involvement of endotoxin and macrophage activation in corticosteroid resistance, and suggested that corticosteroids also exert their beneficial effects through activity on bronchial smooth muscle [60]. "Omics" technologies developments, with data comparison and validation, will lead to the integration of composite signature biomarkers in phenotyping asthma and improvements in our understanding of asthma. Ultimately, breakthroughs in asthma treatment may be reached through the development of innovative targeted therapies [12, 60].

Non-invasive procedures for biomarker analysis form the backbone for day-to-day clinical asthma management. However, invasive tests may provide important information to phenotype and direct therapy in patients with severe refractory asthma [88]. These techniques bring significant additional knowledge in asthma research that needs to be integrated with non-invasive procedures outcomes to allow truly innovative steps in biomarker discovery for asthma management.

6. Asthma phenotypes based on cluster analyses

In general, milder asthma phenotypes respond well to standard therapy with corticosteroids (with or without long-acting beta2-agonists), while those with more severe disease urged the development of new therapeutic modalities. To enable the development of effective (targeted) therapies, it is crucial to understand the pathophysiological mechanisms driving these subsets of asthmatic patients. Haldar et al. performed a cluster analysis on baseline data of 184 patients with mild to moderate asthma coming from different general practitioners (GP) and baseline data of 187 patients with refractory disease from specialist settings [3]. Additionally, a third dataset comprised baseline and longitudinal data of 68 patients with refractory disease followed for 12 months. Hierarchical cluster analysis revealed five different clusters, with some overlapping features between patients from GP and specialist origins. Most importantly, patients with concordant symptoms and (eosinophilic) inflammation (based on sputum analysis) were mostly coming from GP and were characterised by overall milder, often atopic, well-controlled disease, with a benign disease course. Alternatively, patients with uncontrol-

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led disease, characterised by either discordant symptoms (i.e. many symptoms, little airway eosinophilia or non-eosinophilic inflammation) or discordant inflammation (few symptoms, prominent airway eosinophilia) mostly originated from the specialist settings. Commonly found confounders consisted of obesity and non-compliance. Overall, these findings supported a symptom-guided management for mild-moderate "concordant"-type asthma, while "discordant"-type refractory asthmatics might benefit from inflammation-guided therapy [78].

Using unsupervised hierarchical cluster analysis in a group of 726 patients from the Severe Asthma Research Program (SARP) revealed five distinct clinical subphenotypes within this population [4], showing some overlap with the findings by Haldar et al. [3]. The results of both cluster analysis studies underscore disease heterogeneity, even in subsets of patients with similar clinical characteristics, with potentially different pathophysiological and immunological mechanisms, requiring different therapeutic approaches.

Further analysis into the molecular mechanisms underlying different asthma phenotypes revealed at least two distinct subsets with a "Th2-high" and a "Th2-low" profile, respectively [5], based on the expression of IL-13 inducible airway epithelial genes (POSTN (periostin), CLCA1 (chloride channel regulator 1) and SERPINB2 (serpin peptidase inhibitor clade B, member2)) as previously described by this research group. Not unexpectedly, patients with Th2-driven asthma responded well to inhaled corticosteroids while those with a "Th2-low" profile did not. Hence, there is an urgent need for effective therapeutic options for "Th2-low" asthmatic patients that appeared to comprise approximately 50% of the study population, and hence, in reality may be larger than originally thought.

Additionally, these findings urged phenotyping of patients (i.e. including an adequate target population) and/or using an appropriate disease model [8], for adequate interpretation of effectiveness data in targeted intervention studies. So far, several applicable (surrogate) biomarkers have been validated to phenotype potential responders and to monitor the effects of currently available (or under development) targeted therapies, i.e. anti-IgE, and Th2-pathway targeted therapies (anti-IL-5, anti-IL-4 and anti-IL-13) [86]. Presently, biomarkers including blood eosinophils, FeNO and serum periostin thus moved the first steps to personalised medicine [87]. Further insight into the heterogeneity of Th2-driven/type 2 asthma, "Th2-low" subsets, as well as further refinement of sensitive (composite) biomarkers should be considered the next steps in this promising direction to optimise and personalise asthma management.

7. Conclusions

The complex heterogeneity and dynamics of asthma with varying response to standard treatment is driving the search for distinct asthma phenotypes and endotypes. While inhaled corticosteroids can effectively control asthma, therapeutic responses are individualised (though clinical manifestations may match), can be incomplete in a significant number of patients and no curative treatment exists.

In this setting, biomarkers are needed to innovate asthma management. As indicators of pathophysiologic processes or pharmacologic responses, biomarkers can be useful for asthma diagnosis and phenotyping, prediction of future risk or treatment selection or evaluation of response. Non-invasive sampling has the advantage of being patient-friendly and allowing repeatable measurements across all age and severity groups. More direct airway or distant assessment non-invasive sampling and analysis are currently possible, yielding molecular, cellular, functional and imaging potentially clinically useful biomarkers.

For the promise of delivering valuable new biomarkers to the clinic to come forward, it is mandatory that standard optimised procedures are set for sample collection and analysis, and that resulting data are critically processed, explored and cut-off values are well-defined. This will allow comparison of results and replication, with external validation in different population settings.

Though relevant single biomarkers have been found in asthma, increasing evidence shows that biomarker panels do better and composite signatures may indeed soon be integrated in phenotyping/endotyping of asthma. Multiscale, high-dimensional biological, together with standard clinical measures are adding new relevant knowledge. This systems medicine approach is helping to generate new hypotheses and (re)discover pathways and related biomarkers, linking phenotypes to endotypes and ultimately leading to truly innovative treatments for patients with asthma syndromes.

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3. Metabolómica: perspectivas de aplicação na clínica pediátrica

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METABOLÓMICA: PERSPECTIVAS DE APLICAÇÃO NA CLÍNICA PEDIÁTRICA

Judit Morello, Helena Pité e Emília Monteiro

Enquadramento e Definições

A complexidade e os diversos fenótipos do desenvolvimento e das doenças crónicas têm revelado as limitações dos diagnósticos baseados em parâmetros laboratoriais únicos que avaliam vias metabólicas lineares. Para ultrapassar esta limitação surgiu a biologia de sistemas que se caracteriza pela utilização da bioinformática na integração de múltiplas variáveis em simultâneo e na criação de algoritmos que identifiquem fenótipos patológicos. As abordagens metodológicas cujas designações terminam com o sufixo "ómica" são componentes da biologia de sistemas e emergem no final do século XX, após a sequenciação do genoma humano, com o propósito de obter uma abordagem holística das moléculas que compõem um organismo.

As "ómicas" abrangem níveis de complexidade crescente e interdependente, que incluem, entre outras: - **a genómica e a transcriptómica** que examinam os genes (ADN) e a sua expressão (ARN), respectivamente; - **a proteómica** que analisa proteínas; e - **a metabolómica** que quantifica os produtos intermédios ou finais das vias metabólicas.

Admite-se que a metabolómica é, actualmente, a área das "ómicas" mais desafiante para a prática clínica em pediatria por diversas razões. Concretamente:

1. A genómica e a proteómica reflectem apenas o "potencial" para que um evento biológico ocorra e carecem de validação fenotípica. A metabolómica é a "ómica" mais próxima do fenótipo.
2. É consensual que a genómica mudou o paradigma do diagnóstico em neonatologia mas teve escasso impacto na identificação de terapêuticas inovadoras. Os tratamentos existentes continuam a ser dirigidos à correção das vias metabólicas e não dos genes.
3. A epigenética (definida como o estudo das modificações do genoma, que podem ser herdadas pelas próximas gerações, mas que não alteram a sequência do ADN) veio salientar a importância da regulação metabólica do ADN (por ex. metilação) e das proteínas que o envolvem (por ex. modificação das histonas).
   A metabolómica monitoriza directamente esta regulação.
4. O número de metabolitos, comparativamente com o número de variantes nos genes e nas proteínas, é mais reduzido e mais fácil de abordar.
5. As respostas metabólicas podem ocorrer e ser medidas segundos após um estímulo, enquanto a alteração da expressão de um gene e/ou a síntese de uma proteína pode demorar horas ou até semanas.

Apesar da importância da metabolómica, as diversas abordagens "ómicas" são complementares, sendo desejável que possam ser integradas. De facto, esta é já a tendência de alguns dos estudos na área da pediatria (1).

A metabolómica inclui abordagens "dirigidas" ou "globais". As primeiras referem-se à medição simultânea de um conjunto de metabolitos previamente selecionados que integram uma ou mais vias metabólicas de interesse, com base em hipóteses previamente formuladas.
A "metabolómica global" consiste numa abordagem independente de hipóteses formuladas *a priori*, que procura identificar o maior número de metabolitos possível numa única análise. Procura-se, através da análise bioinformática dos dados obtidos, identificar biomarcadores inovadores ou vias metabólicas alteradas não conhecidas, levantando novas hipóteses baseadas exclusivamente em características biológicas metabólicas.

A metabolómica aplicada à Medicina pode permitir identificar e quantificar tanto metabolitos individuais como "assinaturas" compostas de vários metabolitos. Estas últimas consistem no conjunto de vários metabolitos que são combinados e integrados num algoritmo único, interpretado de forma unificada. As "assinaturas" são assim designadas por se assemelharem às vulgares assinaturas, reconhecíveis apenas no seu todo e por poderem contribuir para uma caracterização (metabólica) mais personalizada de cada indivíduo. As "assinaturas" metabólicas podem ser comparadas a um código de barras, que no seu conjunto fornece informação relevante mas cujos componentes individuais (cada "barra", isto é, cada metabolito) podem não ter significado (2).

Aspectos metodológicos e limitações

Os vários aspectos metodológicos envolvidos num estudo metabolómico estão resumidos na Figura 1.

A metabolómica utiliza actualmente tecnologias analíticas de elevada sensibilidade e especificidade como a cromatografia gasosa (GC) ou líquida (LC) associada a espectrometria de massa (GC-MS ou LC-MS) e a espectroscopia de ressonância magnética nuclear (NMR).

As plataformas utilizadas em metabolómica apenas identificam espectros de moléculas cuja separação foi obtida com base na sua carga/massa (espectrometria de massa, MS) ou nas propriedades magnéticas de átomos como ^{1}H ou ^{13}C (NMR). A identificação dos metabolitos pode ser feita, posteriormente, através da utilização de bases de dados, como por exemplo a Human Metaboloma Database Metabolite[1], que associam espectros de MS e de NMR a metabolitos específicos e a vias metabólicas. Estas bases de dados incluem, também, referências a concentrações dos metabolitos em diferentes fluídos biológicos.

[1] http://www.hmdb.ca

A identificação exacta de quais os metabolitos presentes na amostra não é estritamente necessária para, por exemplo, encontrar diferenças entre duas populações. É possível identificar espectros que são distintos entre duas ou mais populações ou grupos de doentes. Esses perfis, avaliados no seu conjunto, podem ser utilizados e validados, mesmo sem estarem identificados todos os metabolitos que dele fazem parte.

As análises metabolómicas podem aplicar-se a qualquer tipo de amostras biológicas (urina, plasma, soro, saliva, ar exalado, fezes, líquido sinovial, tecidos, etc). Ao poderem ser aplicadas a amostras recolhidas por métodos não invasivos, como a urina, e ao produzirem informação metabólica abrangente tornam-se particularmente atrativas e promissoras na neonatologia e pediatria.

Contudo, a aplicação da metabolómica à pediatria está limitada pela variação dos metabolomas ao longo da idade e por não ser extrapolável dos adultos. Em contrapartida pode ser utilizada para avaliar como a dieta ou a exposição ambiental afetam o desenvolvimento pré e pós-natal.

A metabolómica é, para já, uma abordagem assente em tecnologia muito sofisticada, que requer equipamentos caros e recursos humanos altamente diferenciados. As bases de dados de metabolitos não são suficientemente abrangentes e ainda não é conhecido, para a maioria dos metabolitos, o limite entre o normal e o patológico.

A metabolómica na população pediátrica

A aplicação da metabolómica na prática clínica pediátrica é para já limitada à metabolómica dirigida, ou seja, à utilização das metodologias analíticas sofisticadas ao rastreio de doenças hereditárias do metabolismo no recém-nascido já conhecidas, mas que ainda não é acessível por técnicas mais simples. Contudo, a investigação clínica pediátrica na metabolómica global tem crescido exponencialmente nos últimos 10 anos. No quadro 1 listam-se as áreas onde a investigação tem sido mais ativa.

Na medicina fetal, as evidências mais interessantes passam pela identificação de "assinaturas" metabolómicas urinárias das grávidas, que mostraram ser preditivas do crescimento fetal. Acresce a possibilidade de intervenção precoce em fatores de risco modificáveis como o estilo de vida das grávidas, por forma a alterar o metabolismo materno e assim reduzir o risco de doença no recém-nascido (3).

Os resultados da metabolómica em neonatologia foram recentemente compilados por Noto et al (4). Os autores concluem que a metabolómica é particularmente importante para o diagnóstico precoce de patologias graves mas também para a redefinição de saúde e doença.

Nas crianças e adolescentes, as áreas de investigação metabolómica mais desenvolvidas dedicam-se ao estudo de doenças como a asma e a diabetes. A prevalência da asma na população pediátrica e o seu impacto, a diversidade de fenótipos e de prognóstico e as limitações das terapêuticas farmacológicas são alguns dos factores que justificam o maior desenvolvimento desta área. Há resultados encorajadores na aplicação da metabolómica, em tempo real e com dispositivos

portáteis, dirigida à análise de compostos orgânicos voláteis (5,6) e a evidência atual suporta que "assinaturas" compostas de biomarcadores podem ser mais úteis na fenotipagem da asma e na selecção de tratamentos personalizados, comparativamente a estratégias baseadas em biomarcadores únicos (7). Adicionalmente, a abordagem metabolómica global pode contribuir para a estratificação de doentes de acordo com o seu perfil metabólico e não apenas com base em características clínicas observáveis. Contudo, a compilação dos estudos de metabolómica global na asma em amostras líquidas não invasivas (Quadro 2) ilustra uma das suas maiores limitações: a diversidade dos perfis nas diferentes populações estudadas e a dificuldade na interpretação dos seus resultados. Estima-se que possam ser necessários ainda mais alguns anos até a identificação de perfis metabólicos com impacto na caracterização fenotípica e tratamento da asma.

A metabolómica da diabetes tipo 1 (DT1) tem revelado aspectos curiosos. As alterações da regulação do metabolismo dos lípidos e aminoácidos precedem a seroconversão/autoimunidade pancreática (8). Os marcadores de atividade da flora intestinal, quantificados nas fezes, na urina ou no plasma, são diferente nas crianças com DT1 (9,10).

A investigação metabolómica em nefrologia pediátrica tem-se focado particularmente na descoberta de novos biomarcadores com potencial como alvos terapêuticos (11).

O interesse da metabolómica e da metabonómica para a nutrição infantil já em 2014 tinha sido alvo de uma revisão que salienta a sua importância para a identificação, a curto prazo, de marcadores de estado nutricional e a longo prazo para a personalização da dieta na neonatologia e pediatria (12).

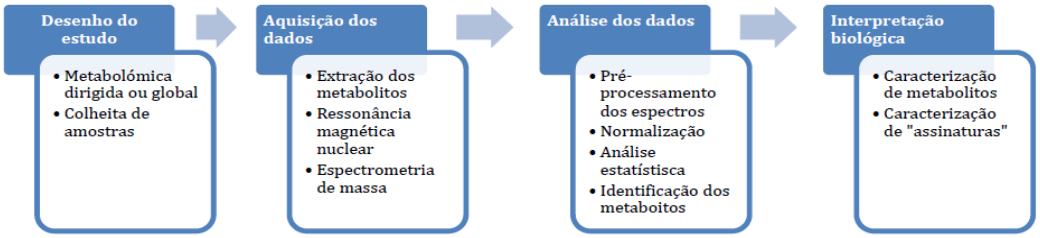
Estudos mais recentes confirmam o potencial da metabolómica para a optimização da composição dos leites (13,14).

A informação sobre a aplicação da metabolómica aos efeitos dos fármacos em pediatria é ainda muito pouco expressiva.

Conclusão

A metabolómica é uma área de investigação em grande expansão em muitos campos da neonatologia e pediatria, com particular interesse para as amostras biológicas não invasivas. O exemplo da asma, onde os resultados são abundantes, demonstra que há já uma pletora de dados que perspectivam a potencial definição de perfis discriminativos a partir do ar exalado, saliva e/ou urina.
A crescente padronização no desenho dos estudos de análise metabolómica e na sua interpretação é mandatória para a validação dos resultados em diferentes populações e a sua utilização futura na prática clínica.

Figura 1. O processo metodológico nos estudos metabolómicos (adaptado de Hanna e Brophy, 2015) (11).



Quadro 1. Áreas de investigação metabolómica em Pediatria.

Medicina fetal	**Restrição do crescimento**
	Exposição a agentes infecciosos
	Idade gestacional
	Peso ao nascer
	Prematuridade
Neonatologia	**Asfixia perinatal**
	Erros do metabolismo
	Fibrose quística
	Nutrição
Pediatria	**Agressividade**
	Anorexia nervosa
	Asma
	Autismo
	Défice de atenção/hiperatividade
	Diabetes
	Displasia broncopulmonar
	Doença celíaca
	Doenças inflamatórias intestinais
	Enterocolite necrosante
	Espondilite anquilosante
	Fibrose quística
	Função renal
	Defeitos congénitos cardíacas
	Microbioma intestinal
	Nutrição e malnutrição
	Obesidade
	Obstipação
	Oncologia
	Sepsis
	Susceptibilidade a infecções

Quadro 2. Resumo dos estudos de metabolómica em amostras líquidas não invasivas na asma na população pediátrica.

População (n)	Amostra (Método)	Metabolitos identificados com potencial capacidade discriminativa	Referência
Controlo saudável (25) Asma (33)	EBC (LC-MS)	Metabolito da prostaglandina, prostaglandina D2, leucotrieno C4, ácido 5-hidroxieicosatetraenoico	(15)
Controlo saudável (15) Asma não grave (31) Asma grave (11)	EBC (LC-MS)	Ácido retinoico, deoxiadenosina, calcitriol, 20-hidroxi-prostaglandina F2alfa, tromboxano B2 e 6-ceto-prostaglandina F1alfa.	(16)
Controlo saudável (24) Asma (65)	Saliva Urina (NMR)	Saliva: arginina, aspartato, citrato, taurina. Urina: ácido butírico, ácido glucónico, ácido pantoténico, ácido quinolínico, lisina, pseudouridina.	(Pité et al. - dados não publicados)
Asma controlada com corticosteróides (15) Asma sem resposta a corticosteróides (15)	Urina (LC-MS)	Gamma-glutamilcisteina, cisteína-glicina, ácido 3,6-dihidronicotinico, 3,4-dihidroxi-fenilalanina, 3-metoxi-4-hidroxifenil(etileno)glicol	(17)
Controlo saudável (12) Asma (41)	Urina (LC-MS)	Ácido urocânico, ácido metil-imidazoleacetico, dipéptido isoleucina-prolina.	(18)
Controlo saudável (42) Asma controlada (53) Asma agudizada (20)	Urina (NMR)	1-Metilhistamina, 1-Metilnicotinamida, 2-Oxoglutarato, 3-Metiladipato, 4-Aminohipurato, O-Acetilcarnitina, fenilalanina, triptofano, etc.	(19)
Asma agudizada sob budesonida e salbutamol (69) ou placebo (48)	Urina (NMR)	Urina: cis-aconitato, lactato, 2-deoxyinosina, 3-metilhistidina, ácido 5-hidroxiindoleacético, 2-aminoadipato, glicose, citrulina, homoserina, histamina, alanina, asparagina, glicilprolina, snglicero-3-fosfocolina, sarcosina, ornitina, creatina, creatinina, glicina, isoleucina and trimetilamine N-óxido.	(20)

EBC: Condensado do ar exalado, LC-MS: cromatografia líquida associada a espectrometria de massa, NMR: ressonância magnética nuclear

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4. Metabolomics in asthma: where do we stand?

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REVIEW

Metabolomics in asthma: where do we stand?

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Purpose of review
Metabolomics has been used to uncover the metabolic signatures of asthma, both for biomarker identification and pathophysiologic mechanisms research. We aimed to review recent advances in this field, published since 2016, and discuss these findings implications to future research and application into clinical practice.

Recent findings
Experimental asthma models and clinical studies in both children and adults supported independent metabolic signatures of asthma. Common reported pathways included purine, glycerophospholipid, glutathione, fatty acids, and arginine and proline metabolism. Metabolomics-based studies identified candidate biomarkers related to asthma severity and corticosteroid resistance, and supported the definition of the obesity-related phenotype at the molecular level. A systematic review with meta-analysis and recent prospective studies favored exhaled volatile organic compounds as one of the most promising biomarkers in asthma diagnosis and monitoring.

Summary
Metabolomics has provided unique and novel insights into asthma profiling at the molecular level. Current challenges include procedures standardization and control of potentially confounding variables for external validation. Point-of-care technology developments bring metabolomics closer to clinical practice. In addition to biomarkers identification, relating metabolites to their biologic role will serve as critical foundations for understanding the biology underpinning asthma heterogeneity and for specific-targeted therapies.

Video abstract
http://links.lww.com/COPM/A22

Keywords
asthma, biomarker, composite signature, metabolomics, phenotype

INTRODUCTION

In the last two decades, the 'omics' disciplines have emerged as important tools in medical research. Metabolomics is a postgenomic discipline that combines high-throughput analytic techniques with bioinformatics to provide a comprehensive analysis of metabolites in biological specimens (Table 1) [1▪▪,2▪▪,3,4]. This profiling of metabolites in biofluids, cells and tissues provides an instantaneous snapshot of a biological system status and can enable the detection of composite metabolic signatures or fingerprints of disease. The application of metabolomics technologies can be particularly profitable to study complex, variable and heterogeneous diseases, such as asthma. The complexity of asthma relies on several pathophysiologic mechanisms, which can interact and may not be present in all patients or at all times. Asthma is currently defined by characteristic symptoms patterns and documented variable airflow limitation, usually associated with airway inflammation and hyperresponsiveness [5]. However, the definition of asthma encompasses patients with different traits, namely regarding clinical presentation, related comorbidities, environmental triggers, lung function impairment, inflammation patterns, airway remodeling features, prognosis, and therapeutic responses [5]. Presently, no strong relationship has been found between pathophysiologic characteristics and particular clinical features or treatment response [5,6]. Metabolomics is the 'omics' field that is closest to phenotype expression and is well suited to reflect the genome–environmental interactions occurring in

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KEY POINTS

- New evidence from metabolomics-based experimental animal models and clinical studies extended the data supporting independent metabolic composite signatures of asthma.
- Metabolomics-based studies identified candidate biomarkers related to asthma, asthma severity and corticosteroid resistance, which require validation.
- Metabolomics can provide relevant data for the definition of molecular asthma phenotypes, as exemplified in the obesity-related asthma phenotype.
- Exhaled volatile organic compounds are promising biomarkers for asthma diagnosis and monitoring, and point-of-care devices can bring real-time metabolomics into clinical practice.
- As current challenges are disentangled (mainly dealing with procedures standardization, metabolite identification and external validation), metabolomics future prospects lie on the unique information it provides for biomarker identification and further understanding the biology behind asthma heterogeneity for personalized asthma care.

asthma. While targeted metabolomics approaches aim at quantifying a set of predefined metabolites, untargeted metabolomics has no metabolite identification before sample analysis and provides a broad and unsupervised multiparametric description of metabolites in a given sample (Table 1). As such, it may contribute to unbiased metabolic profiling of asthma. Besides being routinely applied as a tool for biomarker identification, the inherent sensitivity of metabolomics enables the detection of subtle alterations in biological pathways to provide insights into pathophysiologic mechanisms, metabolic changes over time and the effect of interventions [1▪▪]. Nowadays, metabolomics has been mainly used to uncover composite metabolic signatures of asthma. We herein review recent advances in the field, fully published in 2016 and in the first semester of 2017, considering both studies in humans and experimental animal asthma models, and discuss these findings implications to future research and application into clinical practice.

METABOLIC CHANGES IN ASTHMA: NEW EVIDENCE FROM EXPERIMENTAL ANIMAL ASTHMA MODELS

Recent studies have aimed to identify metabolic changes in ovalbumin-induced asthma mice models, using untargeted liquid chromatography-mass spectrometry (LC-MS; Table 2). All these studies have found asthma-associated changes in a variable number of metabolites in serum or plasma, bronchoalveolar lavage fluid (BALF) and lung tissue. Metabolic pathways suggested to be implicated by at least two of these research groups were glycerophospholipid, sphingolipid, fatty acid, purine, and

Table 1. Definitions

Concept	Definition
Metabolism	Chemical reactions that occur within an organism to sustain life. The reactions are globally divided into two categories: catabolism (the breakdown of organic matter to produce energy) and anabolism (the construction of molecules from smaller units).
Metabolite	Substrate and product of metabolism that drive essential cellular functions, such as energy production and storage, signal transducing and apoptosis.
Metabolome	Complement of naturally occurring and exogenous metabolites within biological systems.
Metabolomics	Large-scale study of metabolomes that aims at the universal analysis of low molecular weight (typically <1500 Da) metabolites in biological specimens.
Targeted metabolomics	Metabolomics methods developed and optimized to measure the concentrations of a predefined set of metabolites of interest. These methods allow higher sensitivity and selectivity and may provide analytical validation to results from untargeted analysis.
Untargeted (global) metabolomics	Metabolomics methods aimed at measuring the broadest range of metabolites present in an extracted sample without a-priori knowledge of the metabolome.
Mass Spectrometry	One of the main analytical platform used for metabolomics analysis that measures the masses of molecules and their fragments [mass-to-charge ratio (m/z) of ions that are formed by inducing the loss or gain of a charge from neutral species] to determine their identity. Usually, preceded by a separation approach (e.g. liquid or gas chromatography).
Nuclear Magnetic Resonance	One of the principal analytical techniques used for metabolomics analysis that exploits the magnetic properties of the atom nuclei (absorption and re-emission of electromagnetic radiation) to obtain physical, chemical, electronic and structural information about molecules.

Data from [1▪▪,2▪▪,3,4].

Table 2. Summary of metabolomics analysis and main results in allergic asthma experimental animal models

Authors [reference]	Main aims	Experimental model	Metabolomics analysis and samples	Main results
Quinn *et al.* [7▪▪]	To identify changes in metabolites found in BALF and in plasma in an acute model of experimental allergic asthma.	C57BL6 female mice (8–10 weeks) -OVA-induced asthma -OVA-sensitized -Control (challenged with PBS or OVA; longitudinal evaluation 6, 24, and 48 h after challenge)	Untargeted HPLC-TOF/MS Targeted HPLC-MS (amino acids) Samples: BALF and plasma from the same mouse	Sphingolipid, glycerophospholipid, neurotrophin signaling, arginine and proline pathways were associated with allergic immune responses. An increase in dysregulated BALF metabolites was found earlier to plasma. AHR correlated with urea-1-carboxylate and ornithine; lung eosinophilia correlated with agmatine.
Su *et al.* [8]	To study the overall metabolic changes in serum of allergic asthma mouse model and to investigate the effects of SPA.	BALB/c female mice (6–8 weeks) -OVA-induced asthma -OVA-induced asthma treated with SPA -Control	Untargeted UPLC-Q-TOF/MS Samples: serum	Purine, glycerophospholipid, fatty acid, phenylalanine, fatty acyls, arginine and proline, valine, leucine and isoleucine, glycine, serine and threonine and amino acid biosynthesis metabolism were disturbed in the serum of allergic asthma mice. After SPA treatment, this metabolomics profile of allergic asthma was significantly reversed, closer to control group.
Yu *et al.* [9]	To investigate the overall metabolic changes in plasma of allergic asthma mouse model.	BALB/c female mice (4–6 weeks) -OVA-induced asthma -Control	Untargeted UPLC-Q-TOF/MS Samples: plasma	Purine, sphingolipid, glycerophospholipid, fatty acid, tryptophan and bile acid biosynthesis metabolism were disturbed in the plasma of allergic asthma mice. Purine metabolism was the most prominently influenced metabolic pathway.
Yu *et al.* [10▪]	To provide a comprehensive understanding of the underlying mechanisms of mKG in allergic asthma.	BALB/c female mice (4–6 weeks) -OVA-induced asthma -OVA-induced asthma treated with dexamethasone -OVA-induced asthma treated with mKG -Control	Untargeted UPLC-Q-TOF/MS Samples: lung tissue and plasma from the same mouse	Purine, sphingolipid, glycerophospholipid, fatty acid, tryptophan, arachinodic acid and bile acid biosynthesis metabolism were disturbed in the allergic asthma mice. The changes of these metabolic profiles were improved with mKG and dexamethasone treatments, except few glycerophospholipid molecules.
Chandler *et al.* [11▪]	To test cadmium-dependent metabolic changes in neurotransmitter and precursor metabolites abundances.	C57B16 male mice (8 weeks) -Cadmium-free -Cadmium-fed	Targeted HPLC-MS Samples: lung tissue	Cadmium ingestion increased metabolites in pathways of glutaminergic, serotoninergic, cholinergic, and catecholaminergic receptors in lung tissue. Environmental cadmium found in food increased AHR and disrupted neuronal pathways regulating bronchial tone.

AHR, airway hyperreactivity; BALF, bronchoalveolar lavage fluid; HPLC, high-performance liquid chromatography; mKG, modified Kushen Gancao Formula derived from traditional Chinese herbal medicines; MS, mass spectrometry; OVA, ovalbumin; PBS, phosphate-buffered saline; Q, quadrupole; SPA, surfactant protein A; TOF, time-of-flight; UPLC, ultra-performance liquid chromatography.

arginine and proline metabolism. Furthermore, an increase in dysregulated BALF metabolites was found earlier to plasma [7▪▪]. This is consistent with an early reaction at the lung–air interface after allergen challenge reflected in BALF and later more downstream effects reflected in plasma. Globally, there was a low overlap of dysregulated metabolites in BALF or lung tissue and plasma, with different change trends [7▪▪,10▪]. Overall, these data support the hypothesis that differentially regulated metabolites are detected in plasma, but reinforce this matrix as a surrogate of pathophysiologic processes that occur after allergen challenge in allergic airway inflammation. The metabolic profile in the

experimental allergic airway inflammation models has been shown to be significantly reversed after interventional strategies using surfactant protein A, the modified Kushen Gancao herbal formula derived from traditional Chinese medicine and dexamethasone [8,10▪].

The study by Chandler *et al.* [11▪] is an example of the use of 'omics' to investigate the effects of environmental exposures (Table 2). Using a targeted metabolomics approach combined with transcriptomics and other analytical methods, the authors found disrupted pathways associated with environmental cadmium in food that may contribute to asthma development [11▪].

NEW EVIDENCE FROM STUDIES IN HUMANS

Table 3 summarizes new evidence coming from metabolomics-based studies in humans. Similarly to experimental asthma models, these studies found that several circulating metabolites in asthma differed from those in healthy individuals [12▪,13▪]. Because the metabolome reflects genome–environmental interactions, it may be strongly influenced by pharmacological and nonpharmacological interventions. Therefore, the effects of disease and disease severity on the metabolome need to be distinguished from those effects due to treatment. Reinke *et al.* [13▪] found that levels of endogenous steroid molecules correlated with inhaled corticosteroids (ICS) dose and were further shifted in patients treated with oral corticosteroids. This is consistent with the dose-dependent effect of these drugs on the hypothalamus–pituitary–adrenal axis suppression. Other metabolites, such as prolylhydroxyproline, were also found to correlate with ICS dose [13▪]. Prolylhydroxyproline is a marker of bone collagen degradation and may reflect an increased risk of osteoporosis and bone injury associated with corticosteroid treatment in a dose-dependent way [13▪].

However, asthma-related metabolic changes have been identified in untreated patients with asthma compared to healthy controls [12▪,13▪], supporting independent metabolic composite signatures of asthma. Different asthma-associated metabolites and potentially implicated metabolic pathways were reported, but hypoxanthine coincided in both studies [12▪,13▪]. This adds further evidence to support the role of the purine pathway in asthma, also in agreement with the results from animal studies [8,9,10▪].

Variable expiratory airflow limitation and hyperresponsiveness are hallmarks of asthma. Fixed airflow limitation is a strong predictor of poor asthma outcomes [5]. An association between lung function parameters and metabolic changes has also been described in asthma, both in children and adults [14,15]. These changes included lipid peroxidation metabolites [14], and also previously described pathways, such as glycerophospholipids, linoleic acid and, again, nucleotide-related metabolites [15]. Metabolites related to lipid peroxidation were also associated with exhaled nitric oxide (FeNO) levels and blood eosinophils count in non-obese adult patients with asthma [14].

Metabolomics can also be used to study the metabolic changes induced by drug interventions. A recent study reported the metabolic effects of budesonide and salbutamol in asthmatic children [16].

Finally, Couto *et al.* [17] performed an exploratory study to investigate the effect of a swimming training session on exhaled oxidative stress markers associated with asthma [25,26], in elite athletes with and without asthma. The authors found a decrease in a predefined set of alkanes and aldehydes in exhaled breath after the swimming session in both groups, with and without asthma, which may possibly be related to adaptive responses to exercise in well trained athletes [17].

METABOLOMICS IN DISTINCT ASTHMA PHENOTYPES: THE OBESITY-RELATED ASTHMA EXAMPLE

Besides the global view of metabolic signatures of asthma, metabolomics can provide deeper insights into distinct asthma phenotypes. Epidemiologic and clinical evidence supports the existence of an obesity-related asthma phenotype, affecting asthma severity, control and medication response [27]. However, body mass index is known to affect the metabolome [28,29], and this, therefore, needs to be considered in metabolomics studies. A study in children found that the metabolite niacin, a potent appetite stimulator, was associated with overweight; however, no metabolite in serum could clearly distinguish between overweight children with asthma from normal weight patients with asthma or overweight healthy controls [12▪]. Nevertheless, a more recent study showed that obese adults with asthma presented a specific respiratory metabolic phenotype, suggesting unique underlying mechanisms [18▪▪]. Exhaled breath condensate profiling could discriminate these patients from lean patients with asthma and obese patients without asthma [18▪▪]. Potential confounding variables, from drug use, food or drinks intake to salivary and room air sample contaminations were monitored and external validation of the obtained classification models was performed by evaluating a distinct cohort. The identified specific biomarkers were involved in the methane, pyruvate, and glyoxylate and dicarboxylate

Table 3. Summary of metabolomics analysis and main results from asthma studies in humans

Authors [reference]	Main aims	Participants characteristics	Metabolomics analysis and samples	Main results
Checkley *et al.* [12▪]	To identify a pattern of serum biomarkers unique to children with asthma.	99 children (9–19 years) -50 with asthma -49 control (each group with equal numbers of children with or without overweight) Country: Peru	Targeted LC-MS (broad + 30 lipids) Samples: serum	A combination of 2-isopropylmalic acid and betain strongly discriminated children with asthma and controls. Children with asthma had lower relative concentrations of serum ascorbic acid, 2-isopropylmalic acid, shikimate-3-phosphate, 6-phospho-D-gluconate and reduced glutathione. Niacin concentrations were higher in overweight children.
Reinke *et al.* [13▪]	To determine whether asthma has a metabolic profile and whether this profile is related to disease severity.	76 adults (18–70 years) -54 with asthma 12 mild asthma 20 moderate asthma 22 severe asthma -22 control Country: England	Untargeted LC-MS Targeted LC-MS (eicosanoids, sphingolipids and fatty acids) Samples: serum	Asthma was characterized by a modest systemic metabolic shift related to disease severity, whereas some metabolic differences were influenced by corticosteroid treatment. Relative to controls, metabolic shifts in mild asthma were associated with exogenous metabolites (e.g. dietary lipids), whereas those in moderate and severe asthma might be related to activating the transient receptor potential vanilloid type 1 receptor.
Loureiro *et al.* [14]	To associate urinary metabolomic profile of patients with asthma with clinical parameters and disease severity.	57 adults (mean age 46 years SD 18.0) All with asthma -17 obese -40 nonobese Country: Portugal	Targeted SPME followed by GCxGC-TOF/MS (34 aliphatic aldehydes and alkanes) Samples: urine	Metabolites related to lipid peroxidation were associated with asthma severity, lung function and eosinophilic inflammation in nonobese patients with asthma.
Kelly *et al.* [15]	To identify metabolites and metabolomic profiles that distinguish children with asthma by their degree of lung function.	380 children (4–13 years) All with asthma -236 with AHR -346 with regular asthma treatment Country: Costa-Rica	Targeted 4 LC-MS complementary methods (broad) Samples: plasma	AHR was associated with 91 metabolites, prebronchodilator FEV_1/FVC with 102 metabolites and postbronchodilator FEV_1/FVC with 155 metabolites. Glycerophospholipid, linoleic acid and pyrimidine metabolism were commonly associated with all three characteristics, but distinct metabolites and pathways were also underlined. The corresponding metabolic profiles showed moderate but robust discriminatory ability.
Quan-Jun *et al.* [16]	To investigate the metabolic changes after inhalation of BUD and SALB during acute asthma exacerbation.	117 children All with asthma exacerbation -69 treated with BUD+SALB (mean age 5 years SD 3.1) -48 not treated (mean age 5 SD 2.8) Country: China	Untargeted NMR Samples: serum and urine from the same person	The perturbed metabolites included 22 and 21 metabolites in serum and urine, respectively. These were involved in seven metabolic pathways: arginine and proline, taurine and hypotaurine, glycine, serine and threonine, glycoxylate and dicarboxylate, methane, citrate cycle, and pyruvate.
Couto *et al.* [17]	To investigate the effect of a swimming training session on oxidative stress markers of asthmatic compared to nonasthmatic elite swimmers.	20 elite swimmers (13–24 years) -9 with asthma -11 control Evaluation before/after training session from the same individual Country: Portugal	Targeted SPME fiber followed by GCxGC-TOF/MS (5 aliphatic alkanes and 3 aldehydes) Samples: exhaled breath	In well trained athletes, swimming was associated with a decrease in the evaluated oxidative stress markers independently of the presence of asthma, although a more pronounced decrease was seen in controls.
Maniscalco *et al.* [18▪▪]	To verify whether metabolomics of EBC from obese patients with asthma, lean patients with asthma, and obese patients without asthma could recognize biomarkers for a separate 'asthma-obesity' respiratory metabolic phenotype.	85 adults (30–51 years) -25 obese patients with asthma -30 obese patients without asthma -30 lean patients with asthma (+ 72 adults in the external validation set) Country: Italy	Untargeted NMR Samples: EBC	Obese patients with asthma were characterized by a respiratory metabolic fingerprint fully different from that of patients independently affected by asthma or obesity. This suggests unique pathophysiologic pathways involved in the pathogenesis of asthma in adult obese patients.
Park *et al.* [19▪▪]	To examine differences in metabolic profiles of urine and related biomarkers between CS-resistance and CS-responsive children with severe asthma.	30 children All with severe asthma -15 CS-respondent (mean age 13 years SD 2.7) -15 CS-nonrespondent (mean age 14 years SD 3.3) Country: Georgia	Untargeted LC-MS Samples: urine	Five potential biomarkers were identified from severe asthmatic children with CS resistance. Tyrosine metabolism, degradation of aromatic compounds and glutathione metabolism were suggested to be significant pathways related to CS resistance.

Table 3 (*Continued*)

Authors [reference]	Main aims	Participants characteristics	Metabolomics analysis and samples	Main results
Cruickshank-Quinn *et al.* [20]	To compare eicosanoid levels from matched EBC and saliva in patients with asthma.	107 adults (27–64 years) All with asthma Country: United States of America	Targeted MRM-MS (16 eicosanoids) Samples: EBC and saliva from the same person	Eicosanoids concentrations in saliva varied widely among patients with asthma. Eicosanoids might be present in EBC, albeit at very low concentrations. The overall results may suggest that previously reported eicosanoids levels in EBC could possibly be due to salivary contamination.
van Mastrigt *et al.* [21]	To assess the repeatability of exhaled profiling with a developed spectroscopy method and compare healthy children and children with asthma or cystic fibrosis.	89 children (6–18 years) -39 with asthma -15 with cystic fibrosis -35 control Country: The Netherlands	Developed method: broadband quantum cascade laser spectroscopy: targeted (VOCs) Samples: exhaled breath	Exhaled breath VOCs profiles showed poor repeatability and agreement of the complete profiles. The developed method detected differenced in VOCs profiles in exhaled breath samples between healthy children and children with asthma or cystic fibrosis.
Brinkman *et al.* [22▪]	To determine whether VOCs discriminate between clinically stable and unstable episodes of asthma.	22 adults (21–32 years) All with mild to moderate asthma on ICS treatment Prospective study after ICS withdrawal and reintroduction Country: The Netherlands	Targeted GC-MS and panel of eNose (VOCs) Samples: exhaled breath	Breath profiles by GC-MS and particularly eNose technology could accurately classify and monitor asthma control. Part of the uncovered biomarkers was associated with sputum eosinophils.
Van Vliet *et al.* [23▪,24▪]	To assess whether exhaled VOCs were able to discriminate asthma control in children. To identify a set of exhaled VOCs predictive of asthma exacerbations in children.	96 children (6–18 years) All with asthma Prospective 12 months study Country: The Netherlands	Targeted GC-TOF/MS (VOCs) Samples: exhaled breath	Children with persistently controlled or uncontrolled asthma could be discriminated by a set of 15 VOCs. A set of seven VOCs in exhaled breath could predict asthma exacerbations in children within 14 days after sampling (sensitivity 88%; specificity 75%).

AHR, airway hyperreactivity; BUD, budesonide; CS, corticosteroid; EBC, exhaled breath condensate; eNose, electronic nose; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; GC, gas chromatography; ICS, inhaled corticosteroid; LC, liquid chromatography; MRM, multiple reaction monitoring; MS, mass spectrometry; NMR, nuclear magnetic resonance; SALB, salbutamol; SD, standard deviation; SPME, solid-phase microextraction; TOF, time-of-flight; VOCs, volatile organic compounds.

metabolic pathways [18▪▪]. Loureiro *et al.* [14] also reported distinct urinary metabolic profiles in obese and nonobese adult patients with asthma.

METABOLOMICS IN SEVERE ASTHMA

Severe asthma management is an important clinical unmet need. Metabolic changes related to asthma severity may be useful as specific biomarkers in diagnosis and management. Loureiro *et al.* [14] observed a correlation between oxidative stress-related metabolites in urine and asthma severity score in nonobese adult patients with asthma. Using an untargeted metabolomics approach, a systemic metabolic shift in an asthma severity-dependent way has also been described, and this appeared to be independent of corticosteroid treatment [13▪]. The metabolites contributing to discriminate patients with moderate to severe asthma may be involved in the activation of vagal nerve transient receptor potential vanilloid type 1 receptors, postulated to be implicated in asthma pathogenesis [13▪].

One of the most striking clinical features of severe asthma is corticosteroid resistance. Using metabolomics analysis in urine samples, Park *et al.* [19▪▪] identified five potential biomarkers of corticosteroid resistance in children with severe asthma. Tyrosine and glutathione metabolism were relevant metabolic pathways, whereas other urinary metabolites associated with corticosteroid resistance were related to smoking [19▪▪].

BREATHOMICS ANALYSIS OF VOLATILE ORGANIC COMPOUNDS

Volatile organic compounds (VOCs) in exhaled breath have gained much interest as noninvasive biomarkers in the past years. Gas chromatography-mass spectrometry (GC-MS) is the gold standard for the identification of exhaled VOCs. The sensor-based electronic noses (eNoses) have the advantage to allow point-of-care testing of composite VOCs signals but do not selectively identify separate molecular components. A recent study used a broad-band quantum cascade laser-based spectroscopy method to detect and identify exhaled VOCs, which could discriminate healthy children from children with stable asthma or cystic fibrosis [21]. The combination of fast and relatively easy to use technology offering the possibility of molecule identification

makes such methods attractive, but additional validation and optimization are needed.

Recently, a systematic review with meta-analysis concluded that exhaled VOCs are promising biomarkers for asthma diagnosis [30▪▪]. Several compounds, mainly alkanes, were associated with asthma. Two more recent prospective studies further extended the data of VOCs to asthma monitoring [22▪–24▪]. An ICS withdrawal study in adults showed that loss of asthma control could be accurately discriminated from clinically stable condition by longitudinal monitoring of exhaled VOCs, particularly by eNose technology [22▪]. Furthermore, an association was found between specific VOCs and sputum eosinophilia [22▪]. Van Vliet *et al*. [23▪,24▪] provided longitudinal data in children, identifying sets of exhaled VOCs that could discriminate children with persistently controlled or uncontrolled asthma, and allowed to accurately predict asthma exacerbations within 14 days after sampling, as had been previously suggested [30▪▪,31]. Contrary to VOCs biomarkers, the FeNO level was not associated with asthma control and was not a significant predictor of exacerbations in these studies [23▪,24▪,31]. Composite VOCs molecular signatures may thus capture clinically relevant changes in asthma outcomes and aid asthma management.

CURRENT CHALLENGES

During the past few years, metabolomics has evolved considerably in an attempt to overcome challenges that limit results generalization [1▪▪]. Standardization from sample collection to data processing and interpretation is critical [1▪▪,32,33▪▪,34▪▪]. Several variables may potentially confound the results. Age, sex, circadian rhythm and exercise affect the metabolome and therefore need to be monitored. In addition to being endogenous (i.e., directly produced by the host organism), metabolites can derive from microbiota, and xenobiotic, medication, dietary, ambient air and other exogenous sources [1▪▪,32,34▪▪]. The metabolome also highly depends on the sample source [35]. Invasively collected lung tissue biopsies or BALF provide more direct airway assessment. However, indirect samples such as blood or even the totally noninvasively obtained urine samples may be useful for metabolic biomarker identification and even underlying mechanisms research in asthma [7▪▪,8,9,10▪,12▪,13▪,14–16,35]. Exhaled breath analysis is very appealing, given the noninvasiveness of its collection, easily allowing repeatable sampling that cope with asthma dynamic changes, and potentially reflecting the airways [35]. However, standardized procedures are mandatory in order to generate comparable results using this highly variable matrix; recommendations have been recently updated [36▪▪].

Although unbiased by previously set hypotheses, untargeted metabolomics results will always depend upon the participants/model inclusion criteria, study design, sample procedures and on the analytical technique and its intrinsic analytic coverage. Nuclear Magnetic Resonance (NMR) is highly reproducible and usually requires less sample preparation but has lower sensitivity; LC-MS or GC-MS are highly sensitive and specific but depend on sample preparation that may affect the results. Relevant metabolites may be present at lower molecular concentrations and it is important to avoid bias towards the more abundant molecules [3]. These drawbacks can be reduced in targeted metabolomics by direct sample preparation to highlight the metabolites of interest and with methods such as triple quadrupole [3]. Metabolomics analyses and untargeted metabolomics in particular generate extensive amounts of raw data. The number of metabolites frequently exceeds the number of samples and high correlation and redundancy between metabolites features is frequent. False-positive results tend to be particularly problematic in high-throughput experiments where many variables must be statistically tested. Computational tools are essential to process and interpret these results and agreed standards in this field are essential. Recent advances promoted novel and more user-friendly tools [1▪▪,32,37▪], addressing several issues including strict strategies to limit false discoveries. Standardization promoting programs are currently available for training researchers in the field [1▪▪]. Identification and validation of relevant metabolites is still a major challenge as only a subset of all metabolite features can be positively assigned to a molecular structure in untargeted metabolomics [1▪▪,2▪▪,32]. Actually, the complexity of the different biological samples comprises a high chemical diversity of metabolites, in different concentrations. These features justify the fact that the majority of detected compounds using the common metabolomics high-throughput techniques still remains unknown.

Presently, metabolomics is still time-consuming and expensive, demanding specialized laboratories and, preferably, multidisciplinary experienced staff to perform the analyses and interpret the results. Point-of-care technology that allows selective identification of separate molecular components is under development. These novel user-friendly methodologies, supported by the information acquired from metabolomics studies, are required for analyzing large numbers of samples at the clinical context. Ongoing research investments to bring

real-time metabolomics close to clinical practice include ion mobility spectrometry methods [36▪▪,38▪].

FUTURE PERSPECTIVES FOR DEVELOPMENTS IN ASTHMA

The initial focus of metabolomics has been on biomarker discovery to identify metabolites associated with asthma or asthma traits. Biomarker validation is challenging but necessary in independent cohorts of biological samples to ultimately allow these biomarkers application in clinical practice. Several exogenous factors that may influence the metabolome must be taken into consideration in future studies to reduce inter-individual and intra-individual variability and allow generalization of the data. With procedure standardization and external validation, developing technology to point-of-care for clinical use in asthma diagnosis and monitoring should follow. Several exhaled VOCs associated with asthma are among the most promising biomarkers, yet struggling with these constraints [30▪▪,38▪]. Nevertheless, considering the accuracy of eNose to identify loss of asthma control, together with the association between specific VOCs and sputum eosinophilia [22▪,39,40], the application of metabolomics fingerprints derived from exhaled breath seems promising for distinguishing eosinophilic asthma and for asthma monitoring and management. Composite molecular signatures may perform better than single biomarkers or biomarker panels in the phenotyping of patients with asthma [35]. For instance, breath analysis by eNose has been shown to predict the response to corticosteroids with greater accuracy than sputum eosinophils or FeNO [41]. Further studies addressing metabolomics-derived biomarkers compared to existing validated markers of asthma are needed.

Metabolomics may further be used to define molecular metabolic phenotypes [1▪▪]. These are based on multidimensional, biology-driven clustering, without predefined hypothesis ('unbiased'). Because more than one pathophysiologic mechanism can occur simultaneously involving common molecules, it may be necessary to use clustering methodologies that allow one element to belong to more than one cluster, instead of separate fixed individualized groups. The usefulness of molecular asthma phenotypes has recently been demonstrated through transcriptomics-driven clustering approaches, which identified patients who may benefit from specific agents that target T-helper cell type 2-mediated inflammation and/or corticosteroid insensitivity [42▪,43▪]. The potential of metabolomics to predict and monitor the response to specific-targeted therapies should be exploited.

Pharmacometabolomics comprehensively analyses the metabolites/metabolic pathways altered by drug interventions, providing relevant data on efficacy and safety [44▪,45▪]. This recently emerged field can be used to profile drug metabolism in asthma and might significantly advance the discovery, development and clinical use of therapeutic drugs. Integrating pharmacogenetics and pharmacometabolomics can provide new insights into the interplay of genomic and environmental influences associated with drug response.

Relating metabolites to their biologic role is the next step [1▪▪]. Metabolomics has already generated several hypotheses to be further tested. Coupling mass spectrometry and NMR techniques to study the metabolic profile of different samples from the same individuals longitudinally provides complementary information to define implicated pathways [46▪▪]. Stable isotope-assisted metabolomics can be settled to trace substrates and ascertain the role of metabolites in metabolic pathways [32]. Multilayered approaches that could integrate clinical data with metabolomics, transcriptomics, proteomics, and other 'omics' data acquired from the same samples may provide an opportunity to investigate the system-wide changes in asthma and to step forward into mechanistic understanding [1▪▪,47▪]. The combination of different biofluids and analytical techniques allows a broad perspective of the metabolome, contributing to the construction of the 'omics' pipeline and the comprehensive understanding of the human status. Large scale and integrative projects adopting a systems medicine approach in asthma and other respiratory and allergic diseases are ongoing, including AirPROM, EARIP, MeDALL and U-BIOPRED. Published papers incorporating omics data for molecular fingerprints were mainly based on proteomics and transcriptomics, but results and achievements of these consortia are currently being disseminated [48,49▪▪,50▪▪,51,52]. Ultimately, these insights are leading to new disease classification hypotheses that can help to improve the understanding of underlying pathophysiologic mechanisms and to better personalized disease management.

CONCLUSION

Metabolomics has provided unique and novel insights into asthma profiling at the molecular level. Once current challenges are overcome, composite metabolic signatures may better reflect the complexity and dynamics of asthma and outmatch single biomarkers or even biomarker panels in asthma management. Metabolomics is a key determinant to reflect genome–environmental interaction networks. Integrated in systems medicine approaches,

metabolomics data can contribute to shape molecular asthma phenotypes, ultimately unraveling specific endotypes for more efficient diagnostics and therapeutics into personalized asthma care.

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Conflicts of interest

There are no conflicts of interest.

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- ■ of special interest
- ■■ of outstanding interest

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