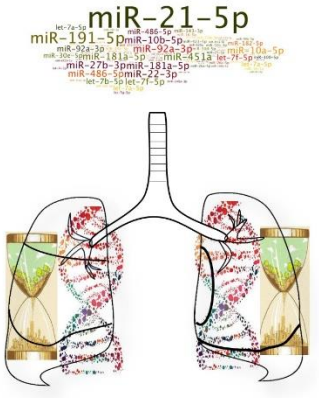

Exhaled breath condensate miRNAs as potential biomarkers to identify and endotype asthma in school-aged children

Ana Francisca Soares de Castro Mendes
Mestrado Biologia Celular e Molecular
Departamento de Biologia
2018

Orientador
André Miguel Afonso Sousa Moreira, Professor Auxiliar com Agregação, FMUP

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
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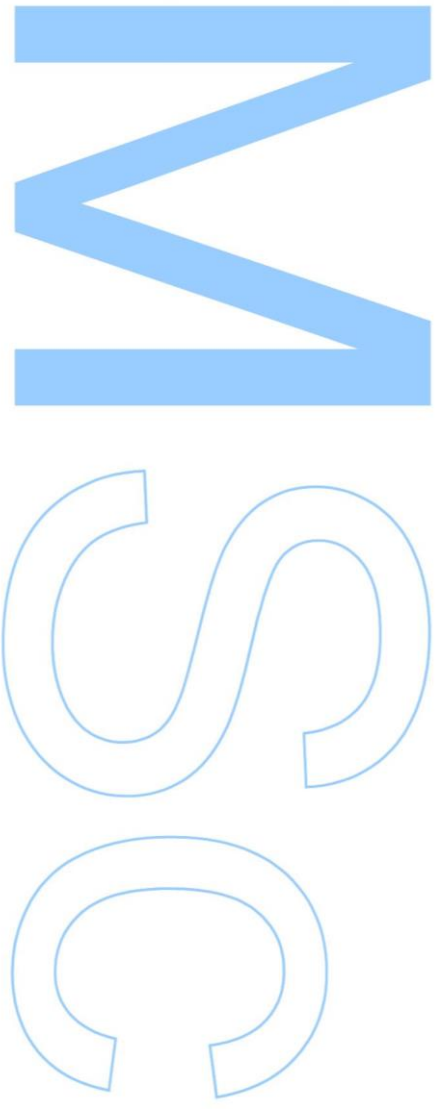

Acknowledgments

Authors gratefully acknowledge the funding by the Project NORTE-01-0145-FEDER-000010 – Health, Comfort and Energy in the Built Environment (HEBE), cofinanced by Programa Operacional Regional do Norte (NORTE2020), through Fundo Europeu de Desenvolvimento Regional (FEDER).

Agradecimentos

Começo por dirigir as primeiras palavras ao meu orientador. Todos os “obrigada” que possa dizer-lhe não são suficientes para agradecer tudo o que fez por mim durante este ano. A sua disponibilidade, dedicação, paciência, ajuda, entusiasmo e motivação foram determinantes para concluir esta etapa. Obrigada por todos os conhecimentos que me transmitiu, por ter confiado e acreditado em mim e por me ter dado a oportunidade de trabalhar e aprender consigo.

Um “obrigada” a todos os meus colegas que também se tornaram amigos. Inês, não tenho palavras para agradecer toda a disponibilidade, interesse, dedicação e amizade que demonstraste ao longo deste período. Obrigada por tudo que me ensinaste. Pedro, das pessoas que mais me fez rir durante esta época. Não imaginas como fiquei mais saudável. Conseguiste sempre fazer-me trabalhar mais, ensinar-me e motivar-me. João e Mariana obrigada por sempre se mostrarem disponíveis em conversar sobre as minhas dúvidas e a solucionar problemas que foram surgindo ao longo desta fase.

Mas esta trabalho é dedicado à melhor mãe do mundo, a minha. Quando for grande quero ser como tu. Obrigada por me ensinares todos os dias a ser mais e melhor, por nunca me deixares desistir (ainda que esta palavra nunca tenha existido no meu dicionário), por me ouvires, aconselhares e ajudares sempre que precisei, por todos os sacrifícios que fizeste e vais continuar a fazer, por seres minha amiga e por mostrares todos os dias o orgulho que tens em mim. Mais um para e por ti.

Agora, ao meu pai…obrigada por sempre me teres motivado e por quereres sempre que desse mais, me esforçasse mais, trabalhasse mais e quisesse mais. Sem dúvida que és o meu exemplo de que nada se consegue sem muitos sacrifícios, muito esforço, muito trabalho e muita determinação. Este também é para ti.

Gonçalo e Carolina, que bom poder ter-vos como irmãos e saber que são os seres humanos mais bonitos que conheci até hoje. Obrigada por todas as vezes que me fizeram rir de mim própria e amenizaram o sentido de responsabilidade de irmã mais velha e por todas as vezes que me fizeram sentir criança no melhor sentido da palavra.

Teams à parte, espero conseguir sempre apoiar-vos e estar presente como vocês estiveram e fizeram nesta fase.

Para a Rosita do meu coração, um enorme obrigada por me fazeres desvalorizar o que não tem importância e por sempre conseguires, com toda a tua graça, tornar um assunto sério numa gargalhada. Quando for ainda maior quero ser como tu.

Também um agradecimento a todos os outros familiares que, com orgulho, sempre me motivaram e incentivaram a chegar até aqui e a terminar esta fase.

A todos os meus amigos, em especial destaque às minhas melhores amigas, Benedita, Maria João, Teresa e Catarina por sempre se mostrarem disponíveis, interessadas e preocupadas em que conseguisse atingir os meus objectivos. Um "obrigada" por todas as conversas de horas, com tantas mensagens positivas e cheias de boas energias e que tanto me ajudaram a "distrair".

Ao meu João, o que traz o melhor de mim, não sei por onde começar a agradecer. Se pela tua paciência em ouvir todos os meus desabafos e todas as minhas "crises" e "ataques"; se por, sempre de forma tão disponível, sincera e honesta, me fazeres sorrir; se por me motivares e incentivares todos os dias; ou se pela tua amizade, respeito e dedicação constante para comigo. Por tudo, obrigada.

Resumo

Os microRNAs (miRNAs) são *small non-coding RNAs* que modulam a maioria dos processos biológicos. Desta forma, o perfil de miRNAs nos diferentes estadios da doença é uma poderosa ferramenta de diagnóstico e prognóstico. No entanto, a deteção e a quantificação de miRNAs no condensado do ar exalado (EBC) têm sido pouco exploradas. O objetivo foi avaliar os miRNAs no EBC como potenciais biomarcadores para diagnosticar e endotipar asma em crianças em idade escolar.

A partir de um *cross sectional, nested case control study*, foram selecionadas todas as crianças com asma (n=71) e uma amostra aleatória de controlos (n=115), com idades compreendidas entre os 7 e os 12 anos, de 71 salas de aula de 20 escolas da cidade do Porto. Os participantes foram submetidos a testes cutâneos por picada e espirometria com broncodilatação, medição dos níveis de óxido nítrico (NO) exalado e recolha de EBC. Com base em estudos anteriores, selecionaram-se onze miRNAs que foram *a posteriori* analisados no EBC por *reverse transcription-quantitative real-time PCR*: let7a-5p, miR21-5p, miR126-3p, miR133a-3p, miR145-5p, miR146a-5p, miR155-5p, miR221-3p, miR328-3p, miR-423-3p e miR-1248. A análise de componentes principais foi aplicada para identificar perfis de miRNAs e as regressões lineares e logísticas foram utilizadas para estimar as associações.

Foram encontradas associações positivas e significativas entre o miR-126-3p e o PC 1 com asma e entre o miR-133a-3p e a broncodilatação positiva sem sintomas. O miR-126-3p, o miR-146a-5p e o PC 2 estavam associados com a resposta das pequenas vias aéreas após broncodilatação e os níveis do miR-328-3p com a resposta do FEV1 após broncodilatação. Também foram encontradas associações entre o miR-126-3p e o miR-328-3p e o NO exalado e entre o miR-221-3p e a dificuldade em respirar.

Os miRNAs podem ser medidos no EBC das crianças em idade escolar e poderão ser futuros potenciais biomarcadores de asma, auxiliando na endotipagem da mesma.

Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that modulate almost all biological processes. MicroRNA profiling in disease states constitutes a powerful tool of diagnostic and prognostic value. Detection and quantification of miRNAs in exhaled breath condensate (EBC) has been poorly explored. We aimed to assess miRNAs in EBC as potential biomarkers to diagnose and endotype asthma in schoolchildren.

In a cross sectional, nested case control study, all the asthmatic children (n=71) and a random sample of controls (n=115), aged 7 to 12 years, attending 71 classrooms from 20 local schools were selected. Participants underwent skin-prick testing, spirometry with bronchodilation, had exhaled level of nitric oxide (NO) determined and EBC collected. Based on previous studies a panel of eleven miRNAs were chosen and analyzed in EBC by reverse transcription-quantitative real-time PCR: let7a-5p, miR21-5p, miR126-3p, miR133a-3p, miR145-5p, miR146a-5p, miR155-5p, miR221-3p, miR328-3p, miR-423-3p and miR-1248. Principal component analysis was applied to identify miRNAs profiles and associations were estimated by regression coefficients using linear and logistic regression models.

Positive and significant associations were found between miR-126-3p and PC1 and asthma and between miR-133-3p and positive bronchodilation without symptoms. Furthermore, miR-126-3p, miR-146a-5p and PC 2 were associated with small airways response after bronchodilation while miR-328-3p was associated with FEV1 response after bronchodilation. Associations were also found between miR-126-3p and miR-328-3p and exhaled NO and between miR221-3p and breathing difficulties.

MicroRNAs can be measured in EBC of schoolchildren and might have future potential as biomarkers of asthma, assisting asthma endotype establishment.

Key-words

Exhaled breath condensate, microRNA, children, asthma

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Abbreviations and Acronyms

AGO2: Argonaute 2 protein

BD: Bronchodilation

BD+S+ : Positive bronchodilation with asthma symptoms

BD+S- : Positive bronchodilation without asthma symptoms

BD-S+ : Negative bronchodilation with asthma symptoms

BMI: Body Mass Index

CDC: Centers for Disease Control and Prevention

Cm: Centimeter

Cq: Quantification cycles

DGCR-8: DiGeorge syndrome critical region gene-8

EBC: Exhaled breath condensate

EXP-5: Exportin-5

FEF 25-75: Forced expiratory flow middle portion of FVC

FeNO: Fractional concentration of exhaled nitric oxide

FEV1: Forced expiratory volume in the first second

FVC: Forced vital capacity

IL: Interleukin

Kg: Kilogram

LNA: Locked nucleic acid

miRNA: MicroRNA

ncRNAs: Non-coding RNAs

NF-kβ: Nuclear factor κβ

NO: Nitric oxide

Nt: Nucleotides

PC: Principal component

PCA: Principal component analysis

PCR: Polymerase chain reaction

Ppb: Parts per billion

qPCR: Quantitative PCR

RISC: RNA-induced silencing complex

RT: Reverse transcription

sncRNAs: Small noncoding RNAs

SPT: Skin prick test

TNF-α: Tumor necrosis factor alfa

VOC: Volatile organic compound

UTRs: Untranslated regions

1. Introduction

1.1. Asthma

The word asthma is derived from the Greek, meaning a short-drawn breath, hard breathing, or death rattle, and thus was, at the outset, a term used to describe a complex of symptoms rather than a specific disease entity (Pavord, Beasley, Agusti et al., 2018). It was first identified as a disease associated with airway (Pavord, Beasley, Agusti et al., 2018) and the Global Initiative for Asthma, describe asthma as a heterogeneous disease, usually characterized by chronic airway inflammation that 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 (Global Initiative for Asthma, 2018). As such, this disease presents differences in severity, comorbidities, natural history, airway obstruction, airway inflammation, airway remodeling and medication response and is associated with many different causes and pathophysiologic pathways (Anderson, 2008; Lötvall, Akdis, Bacharier et al., 2011; Wesolowska-Andersen & Seibold, 2015; Bonnelykke & Ober, 2016).

Asthma is a very common chronic condition in childhood, adolescence and adulthood (Akdis, 2013; Holgate, Wenzel, Postma et al., 2015). The prevalence is higher in countries with high gross domestic product and has increased in most parts of the world over the past few decades and is still increasing in developed western countries (Holgate, Wenzel, Postma et al., 2015). The onset of asthma symptoms is more expected in the first few years of life (Sears, 2015). About 30%-50% of preschool children experience episodes of wheezing. Of these, less than half will have continuing childhood asthma and an estimated 30%-70% of asthmatic children will overcome their symptoms, showing a considerable variability in the course of asthma (Sears, 2015; McGeachie, Davis, Kho et al., 2017). Early childhood and early adulthood asthma and wheezing symptoms are more common in males than females, but males experience asthma remission at a higher rate, and females acquire asthma more often in these age periods, having a higher incidence and persistence of symptoms (Holgate, Wenzel, Postma et al., 2015; Sears, 2015). The development and severity of asthma are driven by strong

genetic and environmental factors and are influenced by immunological and individual (age and sex) factors (Akdis, 2013; Holgate, Wenzel, Postma et al., 2015).

Asthma is a complex and multifactorial disease influenced by multiple elements, being its heterogeneity the result of specific phenotypes and endotypes which are achieved from variations in genetic, physiologic, and immunologic mechanisms that may be influenced by distinct environmental exposures (Casale, 2017) and other factors, such as, body mass index (BMI) and nutrition (Anderson, 2008; Lötvall, Akdis, Bacharier et al., 2011; Bonnelykke & Ober, 2016) (**Figure 1**). As such, both the cellular component and the inflammatory microenvironment are important in the development of the characteristics that define asthma (Sastre, Cañas, Rodrigo-Muñoz et al., 2017). Further, in a syndrome like asthma, in which symptoms are strongly determined by the interaction with the environment, quantification and characterization of an individual's response to the exposome is crucial (Pavord, Beasley, Agusti et al., 2018). However, genetically, there is no gene capable of reflecting the etiology and heterogeneity of asthma (Kontakioti, Domvri, Papakosta et al., 2014).

Asthma is an inflammatory disorder of the lungs that includes airway inflammation (Kontakioti, Domvri, Papakosta et al., 2014), leading to airway hyperresponsiveness (Akdis, 2013; Holgate, Wenzel, Postma et al., 2015) through the infiltration of cells and the release of potent inflammatory mediators to remodel the airway wall (Akdis, 2013). Remodeling is a critical component of asthma that appears due to altered wound repair response of bronchial wall, with secretion of growth factors that induce airway remodeling during chronic inflammation (Akdis, 2013; Kontakioti, Domvri, Papakosta et al., 2014). Airway remodeling occurs throughout the bronchial tree and involves an hypertrophy of airway smooth muscle, thickening of the subepithelial basement membrane, matrix deposition throughout the airway wall, hyperplasia of goblet cell, angiogenesis, neuronal proliferation and metaplasia of epithelial mucus (Akdis, 2013; Holgate, Wenzel, Postma et al., 2015). Furthermore, airway remodeling increases the thickness of the airway wall and leads to irreversible airflow obstruction and airway hyperresponsiveness, and is associated with increased disease severity (Akdis, 2013). All these changes determine additional increase in the airway resistance and contribute to the worsening of lung function that can be observed in chronic asthma (Akdis, 2013). However, the pathophysiologic mechanisms are different even in patients with similar clinical presentations of asthma (Casale, 2017).

Frequently asthma is described in terms of disease phenotypes and there are many suggested, however the underlying biology remains poorly understood. New subphenotypes and associated causal pathways of asthma are being discovered through the application of non-hierarchical statistical analyses of clinical, physiological and laboratory characteristics (Holgate, Wenzel, Postma et al., 2015). However, there is no strongly correlation between these phenotypes and treatment responses, and different inflammatory pathways explain why therapies are only effective in a subset of patients. Asthma treatments are predominantly anti-inflammatory in their action, such as inhaled corticosteroids (Fajt & Wenzel, 2015) but a few recent monoclonal antibodies against interleukin (IL)-4 receptor, IL- 5 and IL-13 have demonstrated efficacy by improving lung function and reducing exacerbations (Slager, Otulana, Hawkins et al., 2012; Noonan, Korenblat, Mosesova et al., 2013; Yancey, Keene, Albers et al., 2017) (**Figure 1**). However, they were particularly effective when subjects were selected using eosinophil- or type 2 immunity- associated markers such as sputum and blood eosinophils, or the fractional concentration of exhaled nitric oxide (FeNO) (Fajt & Wenzel, 2015).

Th2-associated asthma phenotype includes early onset mostly atopic and allergic and a later-onset eosinophilic phenotype. Furthermore, the clinical phenotype of exercise-induced asthma is also likely to have a Th2 component due to its eosinophilic and mast cell-related profile (Holgate, Wenzel, Postma et al., 2015). Th2 immunity upregulates eosinophilic inflammation and tissue damage and results in mediator release, airway hyperresponsiveness and ultimately, in symptoms (Skloot, 2016). Subjects with allergic asthma demonstrate allergic sensitization (Schatz & Rosenwasser, 2014) and, at the molecular level, show upregulation of genes that encode inflammatory proteins, such as inflammatory receptors (Heffler, Allegra, Pioggia et al., 2017). The Th2 immune processes in the airways of people with asthma start with the development of Th2 cells and their production of the cytokines IL-4, IL-5 and IL-13. This cytokines stimulate allergic and eosinophilic inflammation as well as epithelial and smooth muscle changes that contribute to asthma pathobiology (Holgate, Wenzel, Postma et al., 2015). These phenotype can be induced by triggers, such as allergens and non-allergen-induced (for example, exercise) (Kim, DeKruyff, & Umetsu, 2010) (**Figure 1**).

Non/low-Th2-associated asthma phenotype, includes neutrophilic and obesity-related asthma (Lötvall, Akdis, Bacharier et al., 2011; Kontakioti, Domvri, Papakosta et al., 2014; Skloot, 2016). Non-Th2 asthma is likely to represent a large proportion of all

asthma (Wenzel, 2012). However, in comparison to Th2 asthma, little is understood about this asthma phenotype and their underlying molecular elements. Although non-Th2 asthma patients meet the criteria for asthma, show less airways obstruction and hyperreactivity than people with Th2-asthma (Wenzel, 2012). This asthma phenotype is caused by the combination of other cytokines like IL-17 (Skloot, 2016). Furthermore, it is characterized by increased expression of gene pathways such as IL-1β and TNF-α (Skloot, 2016). The neutrophilic phenotype is less responsive to corticosteroid treatment, being reported as more prevalent in older subjects (Hekking & Bel, 2014). Additionally, neutrophilic airway inflammation may be driven by Th-17 cell-mediated processes (Sastre, Cañas, Rodrigo-Muñoz et al., 2017). Neutrophilia can also co-exist with eosinophilia which identifies the people with the most severe asthma (Wenzel, 2012) (**Figure 1**).

Traditional diagnostic approaches have utilized a classification system in which patients are divided into subgroups according to etiologic diagnosis and treatment (Sastre, Cañas, Rodrigo-Muñoz et al., 2017). Phenotypic characteristics may be easily recognizable, since describes an observable characteristic, but asthma is much more complex. As such, the term endotype was created to cluster asthmatics not only by their phonotypical characteristics but also by the pathophysiological characteristics of the disease (Sastre, Cañas, Rodrigo-Muñoz et al., 2017). In other words, an endotype is a subtype of disease defined by a distinct functional or pathobiological mechanism or by response to treatment (Anderson, 2008; Lötvall, Akdis, Bacharier et al., 2011; Bonnelykke & Ober, 2016) and may explain the clinical presentation, epidemiology, and response to different treatments. Each endotype may include several phenotypes and the same phenotype may be present in more than one endotype (Lötvall, Akdis, Bacharier et al., 2011) (**Figure 1**). Although many endotypes have been proposed, they have not yet been widely accepted. In this context, there is a need to generate new classifications of patients which requires the identification of discriminative cellular and biochemical markers (Bos, Sterk, & Fowler, 2016). As such, the molecular understanding of these endotypes may be useful to achieve a more targeted and personalized approach (Kontakioti, Domvri, Papakosta et al., 2014; Wesolowska-Andersen & Seibold, 2015).

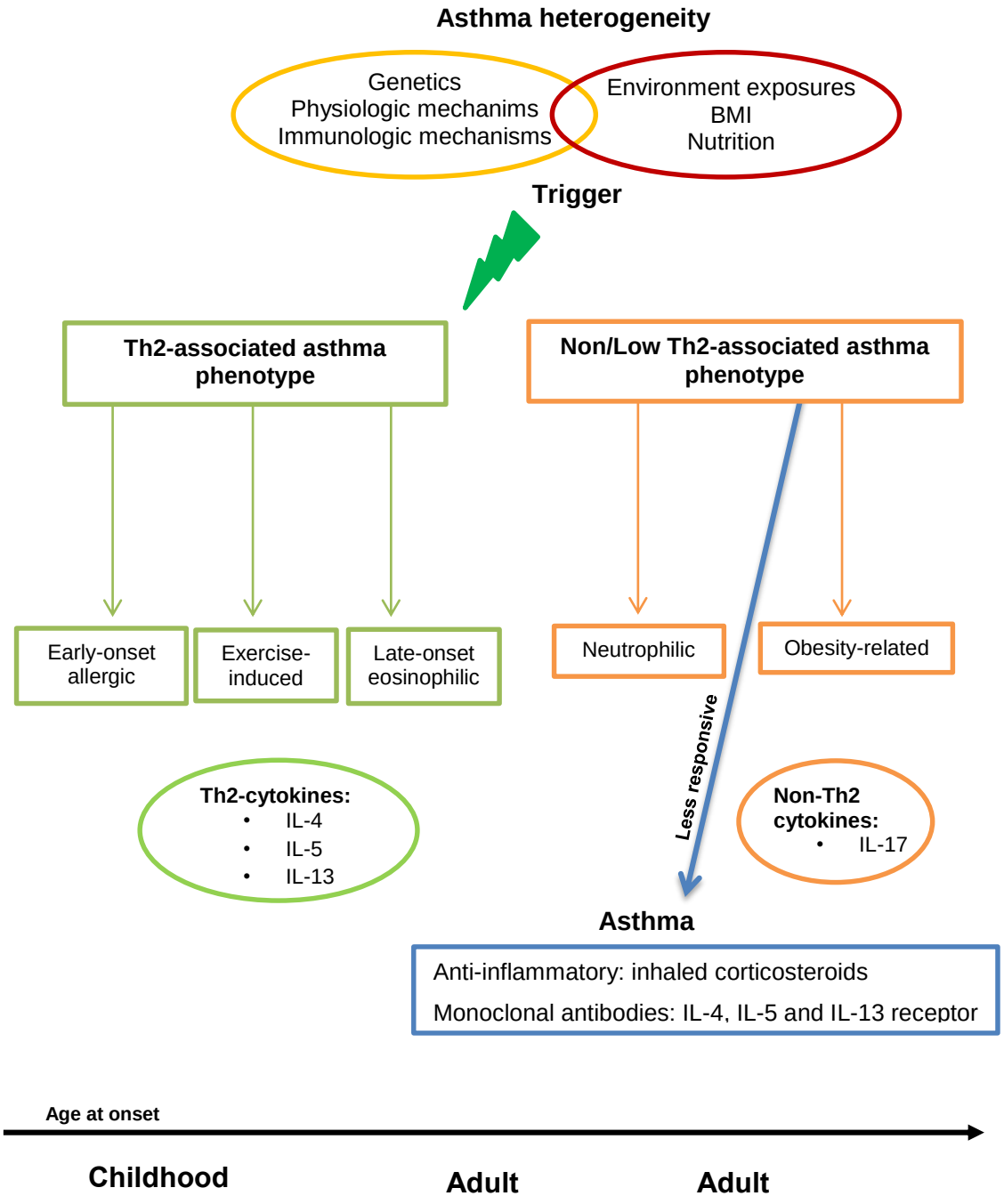


Figure 1. Asthma. The heterogeneity of asthma is the result of specific phenotypes and endotypes which are achieved from variations in different factors. Examples of **Th2-associated asthma phenotypes**, includes early-onset allergic asthma, exercise-induced asthma and late-onset eosinophilic asthma. Non/low-Th2-associated asthma phenotypes, includes neutrophilic and obesity-related asthma. Asthma treatments are predominantly anti-inflammatory in their action, such as inhaled corticosteroids and bronchodilators. Recently, a few monoclonal antibodies have demonstrated efficacy by improving lung function and reducing exacerbations.

1.2. Breathomics

Metabolomics on exhaled air, also called breathomics (Boots, van Berkel, Dallinga et al., 2012), is the analysis of the exhaled breath (Smolinska, Klaassen, Dallinga et al., 2014) that includes the measurement of total breath volatile organic compounds (VOCs) (Carraro, Rezzi, Reniero et al., 2007; Bos, Sterk, & Fowler, 2016), aiming to identify pathways of disease (Bush, 2017). The main component of exhaled breath is condensate vapor, 99.9% of its volume, and water soluble volatile compounds, such as acids and bases, that are highly diluted (Konstantinidi, Lappas, Tzortzi et al., 2015). Further, in addition to VOCs, other different constituents were identified in exhaled breath decades ago (Horvath, Barnes, Loukides et al., 2017). Volatile compounds include acetic acid, formic acid and ammonia, being found in higher concentrations than non-volatile compounds, which include small molecules like sodium ion and large molecules such as immunoglobulins (Hunt, 2007). However many volatile and non-volatile components are present in the breath in scarce amounts, making their detection a challenging task (Horvath, Barnes, Loukides et al., 2017).

Volatile organic compounds are gaseous organic molecules that contain carbon and have a high vapor pressure when in contact with the atmosphere. They are emitted from the fluid phase because they are highly volatile, being released from skin, urine, feces and breath (Munoz, Bustamante, Lopez-Campos et al., 2015; van der Schee, Paff, Brinkman et al., 2015). During exhalation, volatile molecules and water evaporation emerge from airspaces, airways and mouth as gases, and these are then collected in the expiratory airflow (Liang, Yeligar, & Brown, 2012). As such, the VOC content of exhaled breath is a product of the compounds that are inhaled, which are released into the blood and then diffuse into the lungs where they are exhaled (Boots, van Berkel, Dallinga et al., 2012; Smolinska, Klaassen, Dallinga et al., 2014; Schnabel, Fijten, Smolinska et al., 2015), and the reactions that occur between them (Bos, Sterk, & Fowler, 2016)

Several thousands of individual VOCs have been identified (van der Schee, Paff, Brinkman et al., 2015), being originated from both exogenous and endogenous sources (Schnabel, Fijten, Smolinska et al., 2015). Exogenous VOCs are those originated from the environment, which are ubiquitous, appearing from all sources except from the most

inert (Bos, Sterk, & Fowler, 2016). These are released into the bloodstream and then diffused back into the lungs from where they are exhaled (Schatz & Rosenwasser, 2014; Bos, Sterk, & Fowler, 2016; Skloot, 2016). On the other hand, endogenous VOCs are those derived from or affected by metabolism, reflecting host and microbiome metabolism, which includes metabolism of food and drugs that are part of exogenous sources (Boots, van Berkel, Dallinga et al., 2012; Bos, Sterk, & Fowler, 2016). Locally produced compounds diffuse directly into alveoli or the airway lumen along the respiratory tract and systemically produced compounds are derived from the circulation after being originated from metabolic processes elsewhere and dissolving into the blood (van der Schee, Paff, Brinkman et al., 2015). Their concentrations arise from the environment and airway microbiome (Bos, Sterk, & Fowler, 2016) and may change according to health status (Boots, van Berkel, Dallinga et al., 2012; Smolinska, Klaassen, Dallinga et al., 2014).

Breath analysis can be performed in either the gaseous phase as exhaled breath or a liquid phase as exhaled breath condensate (EBC) (Hayes, Haefliger, Harris et al., 2016). Exhaled breath condensate is obtained by cooling exhaled breath through contact with a cold surface or condenser (Horvath, Hunt, Barnes et al., 2005). Samples are collected as fluid or frozen material and analyzed immediately or later for volatile and non-volatile macromolecules (Horvath, Barnes, Loukides et al., 2017). It contains a small admixture of aerosol from the epithelial lining fluid that is produced in the smaller diffusive airways (Sanak, 2016), being the aerosol particles whose contain the non-volatile components (Beck, Olin, & Mirgorodskaya, 2016). These compounds are caught in EBC because exhaled aerosols, which carry metabolites, proteins and even DNA, are dissolved in the condensed water (Mutlu, Garey, Robbins et al., 2001). However, the mechanism of formation of nonvolatile compounds collected in EBC has not yet been fully clarified (Aldakheel, Thomas, Bourke et al., 2016). On the other hand, volatile compounds are present in EBC due to the partitioning between the gaseous and the aqueous phase of the exhaled breath.

Collection of EBC is simple, safe, non-invasive and highly repeatable (Hayes, Haefliger, Harris et al., 2016). The basic method for collecting EBC samples involves a subject breathing into a chilled tube equipped with a one-way valve. Vapor exhaled from the lungs condenses onto the side walls of the tube and is collected into a container (Fox, Spannhake, Macri et al., 2013). Despite the ATS/ERS guidelines, the methodology of EBC is not yet standardized regarding before, during and after condensation. The

ideal condenser dimensions and design, the influence of breathing patterns, cooling temperature, cleaning procedures, condenser tube coating, environmental humidity and temperature, and ambient air pollution on EBC biomarker concentrations, and many other open issues warrant further investigation (Rosias, 2012).

The composition of EBC may be influenced by factors relating to the collection procedure, such as nose breathing, salivary and environmental contamination, and ambient temperature, as well as by constitutive factors relating to the subject, such as age, gender, nutrition and drinking habits, systemic diseases, active smoking and ongoing medication (Moschino, Zanconato, Bozzetto et al., 2015). The human respiratory tract is a route of entry for many environmental and occupational exposures and accordingly, cellular metabolism is altered in disease and volatile and nonvolatile substances in EBC can function as biomarkers, since their concentrations alter in particular pathophysiologic conditions (van der Schee, Paff, Brinkman et al., 2015; Ghio, Madden, & Esther, 2018).

Most of asthma biomarkers used for clinical purposes are sampled in blood, exhaled breath, or urine. Induced sputum remains available only in specialized centers. As such, exhaled breath condensate was developed as an alternative to sputum (Sastre, Cañas, Rodrigo-Muñoz et al., 2017). Compared to the analysis of other body fluid samples such as blood and urine, EBC samples does not produce potentially infectious waste such as needles, bandages, and glassware (Pleil, 2016). As such, the collection of EBC is a unique technique to identify molecular pathways, that may reflect the airway epithelial function (Konstantinidi, Lappas, Tzortzi et al., 2015). Further, it allows the assessment of potential biomarkers of airway inflammation (Mozzoni, Banda, Goldoni et al., 2013; van Mastrigt, de Jongste, & Pijnenburg, 2015), being increasingly used as a tool for biomarker, breath prints, discovery (Cruickshank-Quinn, Armstrong, Powell et al., 2017).

The analysis of VOCs in exhaled breath has become attractive and, nowadays, there has been increased research regarding the development of non-invasive and easy respiratory function tests (Konstantinidi, Lappas, Tzortzi et al., 2015) but several studies have focused on non-volatile organic compounds such as hydrogen peroxide (Munoz, Bustamante, Lopez-Campos et al., 2015) and 8-isoprostane (Peel, Crossman-Barnes, Tang et al., 2017) and more recently miRNAs due to their known relationship to asthma, from diagnose to control. However, many of these biomarkers were identified in other

respiratory diseases, not being specific for asthma. Accordingly, diagnostic biomarkers are needed to appropriately endotype patients and enable the selection of a more personalized therapy. Endotyping may proceed top down, from clinical phenotype to molecular signatures, or bottom up—from molecular signatures to clinical phenotypes and the study of EBC composition seems to be a good place to start for a bottom up approach (Anirban Sinha, Desiraju, Aggarwal et al., 2017).

Breathomics of EBC may provide a key step towards personalized medicine, since it can be potentially discriminatory between “with asthma” and “without asthma”, characterizing asthma endotypes. The final clinical goal of studying EBC is to optimize treatment for patients by taking into account individual patients’ breath characteristics. Additionally, EBC contains miRNAs (Pinkerton, Chinchilli, Banta et al., 2013; A. Sinha, Yadav, Chakraborty et al., 2013), which may be useful for the molecular diagnosis of asthma since its analysis produce findings that express a considerable part of proteomic, metabolic, and genomic fingerprint (Konstantinidi, Lappas, Tzortzi et al., 2015). Furthermore, the collection of exhaled breath in children between 3 and 16 years with asthma is feasible and acceptable with a relatively high sensitivity and specificity (Neerincx, Vijverberg, Bos et al., 2017).

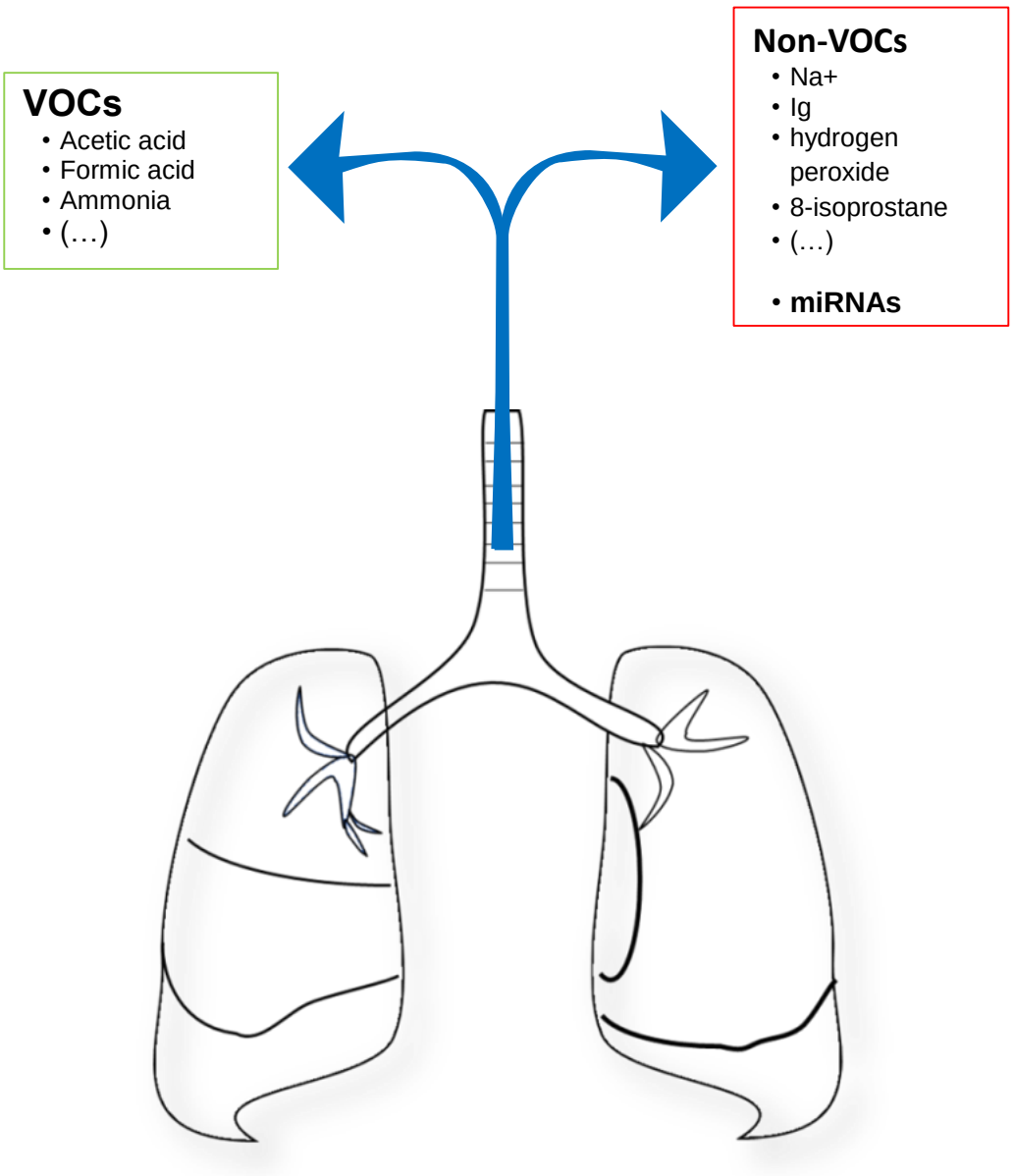


Figure 2. Exhaled breath. Metabolomics on exhaled air can be performed in exhaled breath condensate (EBC). Collection of EBC is a unique technique among lung function tests in terms of identifying molecular pathways, which reflect the airway epithelial function. It allows the assessment of potential biomarkers of airway inflammation, being increasingly used as a tool for biomarker discovery. The analysis of VOCs in exhaled breath has become attractive however several studies have focused on other compounds, like hydrogen peroxide and 8-isoprostane and more recently miRNAs due to their known relationship to asthma, from diagnose to control.

1.3. MicroRNA

Complex diseases have been related to an interaction between genes and environment. Epigenetic mechanisms start with environmental factors, being a link between environmental stimuli and gene expression (Y. Liu, Li, Xiao et al., 2013). Epigenetics studies the heritable changes of a phenotype, including gene expression without any variation in DNA sequence, which means that an epigenetic modification implies an adaptation of genes to environmental alterations (Kabesch, Michel, & Tost, 2010). This modifications are often responsible for triggering disease in susceptible individuals (Norman, Carpenter, Scott et al., 2013). Common epigenetic mechanisms include DNA methylation, histone modifications and the regulatory activity of small noncoding RNAs (sncRNAs), such as miRNAs (Brandão, Guerra, & Mori, 2017; Narozna, Langwinski, & Szczepankiewicz, 2017), which control gene expression (Kabesch & Adcock, 2012; Lovinsky-Desir & Miller, 2012; Alizadeh, Mortaz, Adcock et al., 2017).

Small non-coding RNAs belong to non-coding RNAs, which are divided into housekeeping RNAs and regulatory RNAs (Narozna, Langwinski, & Szczepankiewicz, 2017). Non-coding RNAs (ncRNAs) are powerful regulators of gene expression that influence numerous biological pathways including proliferation, differentiation and apoptosis (Narozna, Langwinski, & Szczepankiewicz, 2017) by altering the expression of hundreds of targets (Dey, Mueller, & Dutta, 2014). Housekeeping RNAs include those involved in ribosomal machinery, such as rRNAs, transfer RNAs, small nuclear RNAs and small nucleolar RNAs, whereas regulatory RNAs include small non-coding RNAs with less than 200 nucleotides and long non-coding RNAs with more than 200 nucleotides (Dey, Mueller, & Dutta, 2014). Although noncoding RNAs, housekeeping RNAs, long noncoding RNAs, and small noncoding RNAs have emerged as important molecules in lung disease, miRNAs are the group more studied (Szymczak, Wieczfinska, & Pawliczak, 2016).

Mature miRNAs are a highly conserved small noncoding single-stranded RNAs (Simonson & Das, 2015) of 18–25 nucleotides (nt) responsible for the post-transcriptional regulation of protein-coding mRNAs (Song, Xu, Ji et al., 2014; Deiuliis, 2016; Shi, Huang, Gu et al., 2016), thus controlling gene expression (Chen, Dai, Ji et al., 2014). The human genome contains hundreds of miRNAs, which are expressed in a tissue-specific manner

and are involved in innumerous cell processes such as in proliferation, apoptosis and differentiation during mammalian development (Kabesch, Michel, & Tost, 2010). Further, they have been identified as an important player in the pathogenesis of many inflammatory diseases including allergy, having several possible modes of action such as Th2 polarization, innate immune cells in inflamed tissue, and chronic inflammation in epithelium and tissue remodeling (Dissanayake & Inoue, 2016; Z. Liu, Zhang, Callejas-Díaz et al., 2016).

An individual miRNA can regulate the expression of several target mRNAs and multiple miRNAs may regulate a single mRNAs (Thomas X. Lu & Rothenberg; Cai, Yu, Hu et al., 2009). As such, miRNAs are regulated by transcriptional activation or inhibition, epigenetic repression, and controlled degradation rates (Mohr & Mott, 2015). This regulation takes place at multiple steps, including their transcription, their processing by Drosha and Dicer, their loading onto AGO proteins and miRNA turnover (Ha & Kim, 2014). Further, miRNA maturation depends on multiple pathways that regulate the biogenesis, stability and/or formation of miRNA-protein complexes that modulate gene expression (Deiuliis, 2016).

In humans, the majority of canonical miRNAs are encoded by introns if noncoding or coding transcripts however some are encoded by exonic regions (Ha & Kim, 2014) (**Figure 3**). Specifically, genomic miRNA sequences can be localized in intergenic regions, driven from polycistronic transcripts that often encode multiple, closely related genes, or in introns of protein-coding genes (Rebane & Akdis, 2013). This polycistronic mode of transcription suggests the involvement of members of the same miRNA family in related pathways (Cai, Yu, Hu et al., 2009). Additionally, miRNAs in the same cluster are generally co-transcribed (Ha & Kim, 2014) and be interrelated, but this situation is not always bidirectional, which means that they could be related between them but not form a cluster (Sastre, Cañas, Rodrigo-Muñoz et al., 2017).

Biogenesis of miRNAs starts with the transcription of miRNAs genes by a RNA polymerase II, generating pri-miRNA transcripts (Peng, Yu, Li et al., 2014) (**Figure 3**). Generally, a typical pri-miRNA consists of a stem of 33–35 bp, a terminal loop and single-stranded RNA segments at both the 5´ and 3´ sides (Ha & Kim, 2014; Simonson & Das, 2015). The transcription of miRNAs is controlled by RNA-Pol II-associated transcription factors and epigenetic regulators (Ha & Kim, 2014).

Following transcription, these primary microRNAs, whether mono or polycistronic are processed by the Drosha-DiGeorge syndrome critical region gene 8 (DGCR-8) complex, also called microprocessor complex (Wahid, Shehzad, Khan et al., 2010; Lorenzo, Loarca, Ivanics et al., 2015) (**Figure 3**), that cleaves the stem-loop and releases a small hairpin-shaped RNA, called pre-miRNA (Sandoval-Bórquez, Saavedra, Carrasco-Avino et al., 2015). Drosha interacts with its co-factor DGCR-8 (Kwon, Nguyen, Choi et al., 2016), a protein that localizes to the nucleoplasm and the nucleolus (Ha & Kim, 2014). The primary role of DGCR8 is to recognize the primary miRNA (Sohn, Bae, Kim et al., 2007). Further, in this complex, Drosha function as the catalytic subunit and determines the cleavage sites by measuring the length of the double-stranded RNA (~11 bp) from the basal junction (Kwon, Nguyen, Choi et al., 2016). This nuclear protein belongs to a family of RNase III-type endonucleases that act specifically on double-stranded RNA, initiating the maturation process by cropping the hairpin to release a small hairpin-shaped RNA of ~65 nt in length, the pre-miRNA (Ha & Kim, 2014).

After Drosha processing, the pre-miRNAs are exported to the cytoplasm in order to complete the maturation (Ha & Kim, 2014) (**Figure 3**). This exportation happens via protein exportin 5 (EXP5) that forms a transport complex with GTP-binding nuclear protein RAN-GTP and a pre-miRNA (Ha & Kim, 2014). In cytoplasm, pre-miRNAs are clived by Dicer, that recognizes the termini of double-stranded RNA using its PAZ domain and determines its cleavage sites by measuring ~22 nt from the terminus (Kwon, Nguyen, Choi et al., 2016), near the terminal loop, liberating a small RNA duplex (**Figure 3**) (Ha & Kim, 2014). This small RNA duplex is subsequently loaded onto an AGO protein to form an effector complex, called RNA-induced silencing complex (RISC) (Ha & Kim, 2014). RNA-induced silencing complex uses the miRNA to direct sequence-specific recruitment of the RISC to its target mRNA (Mohr & Mott, 2015). RISC binds to their target mRNA at the 3' untranslated regions (3' UTRs) in at least a length of 6- to 8-nt (Rebane & Akdis, 2013), leading to the silencing of the target mRNA by direct degradation or repression of its translation (Brandão, Guerra, & Mori, 2017), thus negatively regulating or "repressing" target gene expression (**Figure 3**).

Mammalian miRNAs genes have multiple isoforms, paralogs, that often have identical sequences at nucleotide positions 2-7 relative to the 5' end that are called "seed sequences" (Sastre, Cañas, Rodrigo-Muñoz et al., 2017). Specificity and activity in binding targets have a predominant participation of the 5' region of the miRNA (Cai, Yu, Hu et al., 2009). The interactions between miRNA and mRNA target is preferentially

restricted to the “seed” (Mohr & Mott, 2015). The seed is constituted by ~6 to ~8-nt and it is highly conserved among species and a slight change in sequence, can alter its target spectra (Cai, Yu, Hu et al., 2009). Further, this sequence of ~6nt is decisive in paring with target mRNAs, conferring upon miRNAs promiscuity behavior, reflecting their capability in regulate multiple different miRNAs (Sastre, Cañas, Rodrigo-Muñoz et al., 2017).

In general, mRNA degradation happens when high level of complementary exists between miRNA and target mRNA (“perfect” binding), whereas translation repression takes place when complementary between miRNA and target mRNA is incomplete (“imperfect” bind) (Mohr & Mott, 2015). This “perfect” or “imperfect” bind between miRNAs and mRNA explains why one miRNA can regulate many different targets, causing a specific and a large scale regulation of translation (Simonson & Das, 2015). However, not all functional miRNA interactions involve a reduction in the expression of the target gene. For example, miRNA binding to the promoter can increase target gene expression (Mohr & Mott, 2015).

Micro-RNA expression profiles differ between disease and healthy states (Mohr & Mott, 2015), being crucial for the growth and functioning of the lungs (Heffler, Allegra, Pioggia et al., 2017). Furthermore, the lungs have been demonstrated to have a very distinctive miRNA profile (Heffler, Allegra, Pioggia et al., 2017) which may contribute to the development and progression of asthma in case of abnormal expression (Kabesch, Michel, & Tost, 2010). However, the fact that miRNAs have the potential to target hundreds of mRNAs, creates a challenge in identifying their functional roles (Mohr & Mott, 2015).

The recent discovery of circulating miRNAs in serum/plasma and EBC, in a form that is relatively stable compared to mRNAs and proteins, suggest that miRNAs may be potentially important clinical non-invasive biomarkers for diagnosis, classification, and outcome prediction of certain diseases (Thomas X. Lu & Rothenberg, 2013; Z. Liu, Zhang, Callejas-Díaz et al., 2016), like asthma. In this context, in the search of representative samples derived directly from the airways, analysis in different body fluid samples and in structural cells has been performed (**Table 1**). Further, the heterogeneity that exist between asthmatics both in terms of clinical severity and structural changes of the involved tissues (Heffler, Allegra, Pioggia et al., 2017) creates a need in establishing possible biomarkers for diagnosis or prognosis. Accordingly, diagnostic biomarkers are

needed to appropriately endotype patients and enable selection of a more personalized therapy.

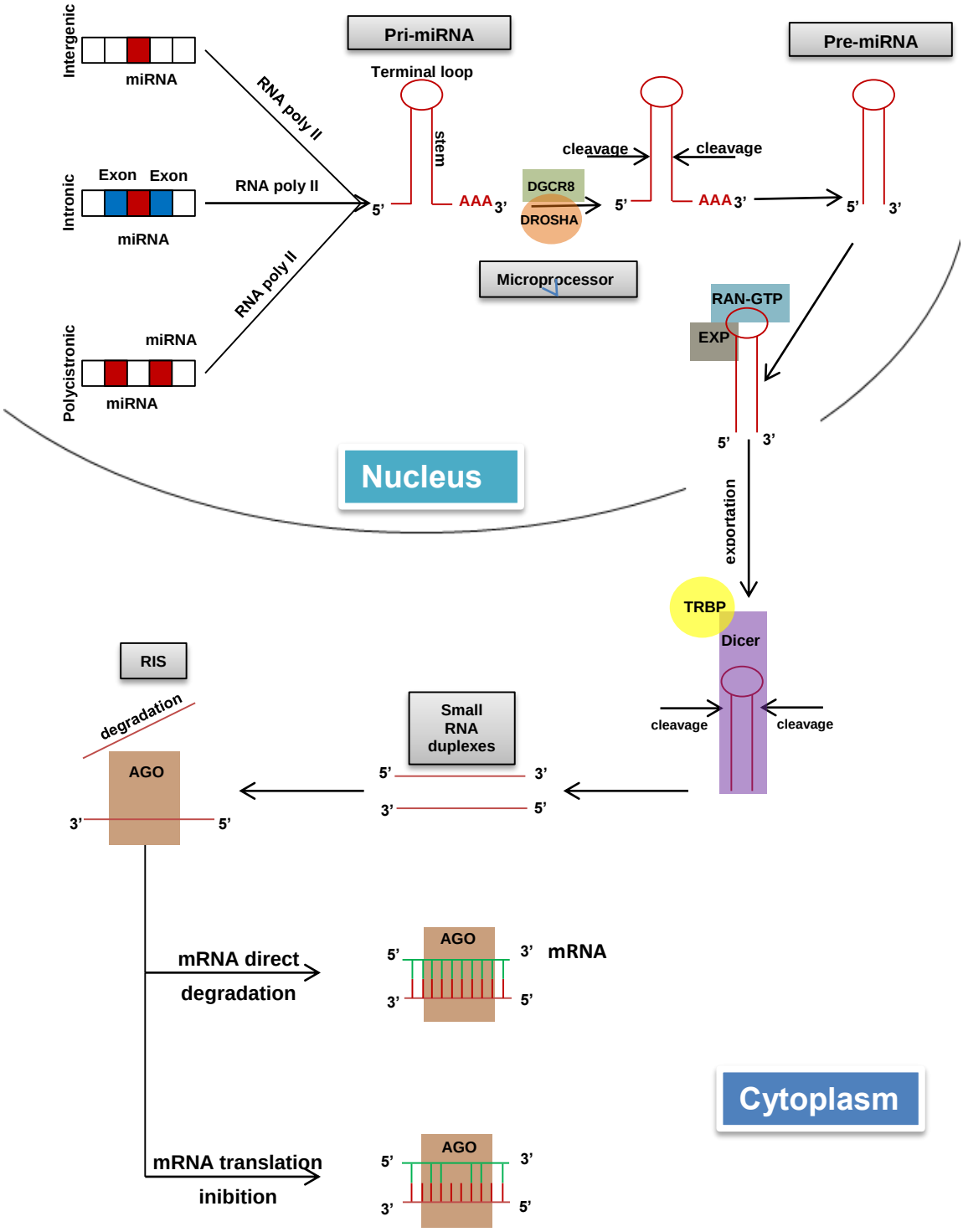


Figure 3. Canonical pathway of miRNA biogenesis in humans. MicroRNA genes are transcribed by RNA polymerase II from intergenic, intronic, or polycistronic loci, originating a long primary transcript, called primary miRNA (pri-miRNA). a typical pri-miRNA consists of a stem of 33–35 bp, a terminal loop and single-stranded RNA segments at both the 5´ and

3´ sides. Following transcription, primary microRNAs, are processed by microprocessor complex, Drosha and DGCR8 co-factor, that cleaves the stem-loop and releases a small hairpin-shaped RNA, called pre-miRNA. After Drosha processing, the pre-miRNAs are exported to the cytoplasm via protein exportin 5 (EXP5) that forms a transport complex with GTP-binding nuclear protein RAN-GTP and a pre-miRNA. Subsequently, pre-miRNAs are cleaved by Dicer, TAR RNA Binding Protein (TRBP), and Protein Activator of PKR (PACT), producing small RNA duplexes. These small RNA duplexes are subsequently loaded onto an AGO protein to form an effector complex, called RNA-induced silencing complex (RISC). RISC binds to their target mRNA at the 3' untranslated regions (3' UTRs) in at least a length of 6- to 8-nt leading to the silencing of the target mRNA by direct degradation or repression of its translation thus negatively regulating or "repressing" target gene expression. RISC binds to their target mRNA at the 3' untranslated regions (3' UTRs). The interactions between miRNA and mRNA target is preferentially restricted to the "seed", the nucleotide positions 2-7 relative to the 5' end.

Table 1. MicroRNAs (miRNAs) expression profiling in human samples

Sample	Population	Expressed miRNAs	Method	Reference
Exhaled breath condensate	10 patients with chronic obstructive pulmonary disease (38.2 ± 15 years), 11 patients with asthma (45.4 ± 13.9 years), and 12 healthy subjects (58.1 ± 5.0 years)	miR-21, miR-133, miR-155 and miR-328 levels were significantly lower in asthmatic adults when compared to healthy control adults	Real-time PCR	(Pinkerton, Chinchilli, Banta et al., 2013)
Sputum	16 allergic asthmatics (22 to 63 years) and 13 healthy controls (25 to 61 years)	significantly lower levels of miR-155 and a trend to lower levels of miR-146 in allergic asthmatic adult group then in healthy control group	Quantitative real-time PCR	(Malmhall, Johansson, Winkler et al., 2017)
	13 patients with asthma (63.3±8.96 years), 31 patients with COPD (52.5±13.2 years), 8 patients with asthma–COPD overlap syndrome	miRNA-145 levels were higher in asthmatic adult patients when compared to healthy control	Quantitative real-time PCR	(Lacedonia, Palladino, Foschino-Barbaro et al., 2017)

	(70.3±8.01 years) and 7 controls (65.2±5.45 years)			
Blood	4 children allergic asthma (4 to 6 years) and 4 children control group (4 to 6 years)	miR-221 levels were upregulated in asthmatic children when compared to healthy control children	Real-time PCR	(Qin, Xu, Mei et al., 2012)
	35 asthmatic patients (41.5 ± 3.25 years), 25 non-asthmatic patients with AR (39.8 ± 3.12 years), and 19 non-allergic non-asthmatic subjects (43.9 ± 2.47 years)	miR-126 and miR-133a levels were upregulated in asthmatic adult patients	Microarray and real-time PCR	(Panganiban, Wang, Howrylak et al., 2016)
	6 never smokers with COPD, 19 current smokers with COPD, 8 asthma patients, and 6	upregulation of miR-145-5p in adult asthmatic patients	microarry	(Wang, Huang, Liang et al., 2016)

	healthy controls			
	347 asthma patients (41.8 ± 13.9) and 172 healthy (29.1 ± 9.4)	miR-146 polymorphisms was associated with airway hyperresponsiveness in Korean adult asthmatics	NA	(Trinh, Pham, Kim et al., 2017)
Lymphocytes	6 children allergic asthma and 6 children control group (4 to 6 years)	miR-221 levels were upregulated in asthmatic children when compared to healthy control children	Microarray and real-time PCR	(F. Liu, Qin, Xu et al., 2012)
Airway biopsies	8 mild-asthmatics (29 ± 3 years) and 8 healthy individuals (20 ± 1 years)	227 miRNAs studied but no differences in miRNA expression were found between mild asthmatics adults and healthy control group	Quantitative PCR	(Williams, Larner-Svensson, Perry et al., 2009)
	34 healthy controls (33.3 ± 1.7 years), 50 patients with allergic rhinitis (33.2 ± 1.4 years), 36 patients with allergic rhinitis and asthma (32.5 ± 1.1	miR-155 levels were upregulated in asthmatics	Real-time quantitative reverse-transcription PCR	(Suojalehto, Toskala, Kilpelainen et al., 2013)

	years), 39 patients with non-allergic rhinitis (33.2 ± 1.5 years)			
	117 middle-aged men with allergic asthma for approximately 20 years and 33 healthy controls	miR-126 and miR-155 were downregulated when compared to control group	Quantitative PCR	(Suojalehto, Lindstrom, Majuri et al., 2014)
Bronchial cells	cells from asthma patients treated with (treatment group, n=19) or without (non-treatment group, n=16) inhaled corticosteroids or from non-asthmatic healthy individuals (normal group, n=12)	miR-21 and miR-126 were increased in asthmatic adult patients, with or without inhaled corticosteroids treatment, after culture with IL-13	Quantitative real-time PCR	(Wu, Wang, Zhu et al., 2014)

NA: not available

2. Aim of the study

Understanding the molecular mechanisms responsible for asthma development, as well as finding a non-invasive disease biomarker, would be fundamental to better monitor and to early diagnose asthma in school-aged children, enhancing prognosis and therapy.

Asthma diagnosis is not easy since commonly there are no correlation between asthma phenotypes and treatment responses.

MicroRNA levels vary greatly among tissues, and it is believed that deregulation of miRNA can contribute to asthma development. Therefore, miRNA in asthma, might have a central role in influencing inflammatory pathways, particularly in EBC produced from pulmonary cells. Therefore, it is of great interest the analysis of miRNAs as a fundamental part of mechanisms of growth and function of the lungs. Further, the lungs have been demonstrated to have a very distinctive miRNA profile.

Globally this thesis aims to develop and validate the use of a non-invasive methodology to identify asthma biomarkers to diagnose and endotype pediatric asthma

Specifically, we will aim to:

- Develop a non-invasive approach for sampling, extracting and profiling miRNA of the airways of children;

- To determine whether exhaled breath condensate miRNAs can help differentiate between asthmatic and healthy children;

- To assess the profile of specific miRNA expression in EBC of asthmatic children as a candidate biomarkers of asthma endotypes.

3. Methods

3.1. Participants and study design

In this nested case-control study, we analyzed the exhaled breath miRNA profile in 71 with and 115 children without asthma. Study population was drawn from a cross sectional survey conducted between January 2014 and March 2015, that enrolled 1602 children aged 7 to 12 years old attending the 3rd and 4th grades from 20 primary schools in Porto, Portugal (Cavaleiro Rufo, Paciencia, Silva et al., 2018). We assessed symptoms, atopic status, airway inflammation and measured lung function. Children not being able to comply with study procedures or that failed to provide an exhaled breath condensate volume of at least 400 µL were excluded (**Figure 1**).

All children that had a positive bronchodilation (BD) defined as at least a 12% and over 200mL increase in FEV1 after inhaling 400 µg of salbutamol; or had self-reported medical diagnosis of asthma with symptoms (wheezing, dyspnoea or dry cough) in the previous year were considered as having asthma. A random sample of the remaining children, stratified for body mass categories, based on a case: control ratio of 1:2, was selected.

Different asthma phenotypes were considered according to previously published phenotypes (Haldar, Pavord, Shaw et al., 2008; Zoratti, Krouse, Babineau et al., 2016): i) allergic asthma, defined by positive skin prick tests (SPT); ii) eosinophilic asthma, defined by exhaled nitric oxide above 35 ppb; iii) obese asthma, defined by overweight or obesity ; iv) treated asthma, defined by current use of anti-asthma medication ; v) symptomatic asthma, defined by current symptoms; vi) positive bronchodilation with asthma symptoms (BD+S+) ; vii) positive bronchodilation without asthma symptoms (BD+S-); and viii) negative bronchodilation with asthma symptoms (NB-S+).

Characteristics of participants and phenotypic characteristics of children with asthma are presented in table 2 and table 3, respectively.

Study was approved by the Ethical Committee of the University of Porto, every procedure was in accordance with Helsinki Declaration, and written consent from children's legal guardians was obtained.

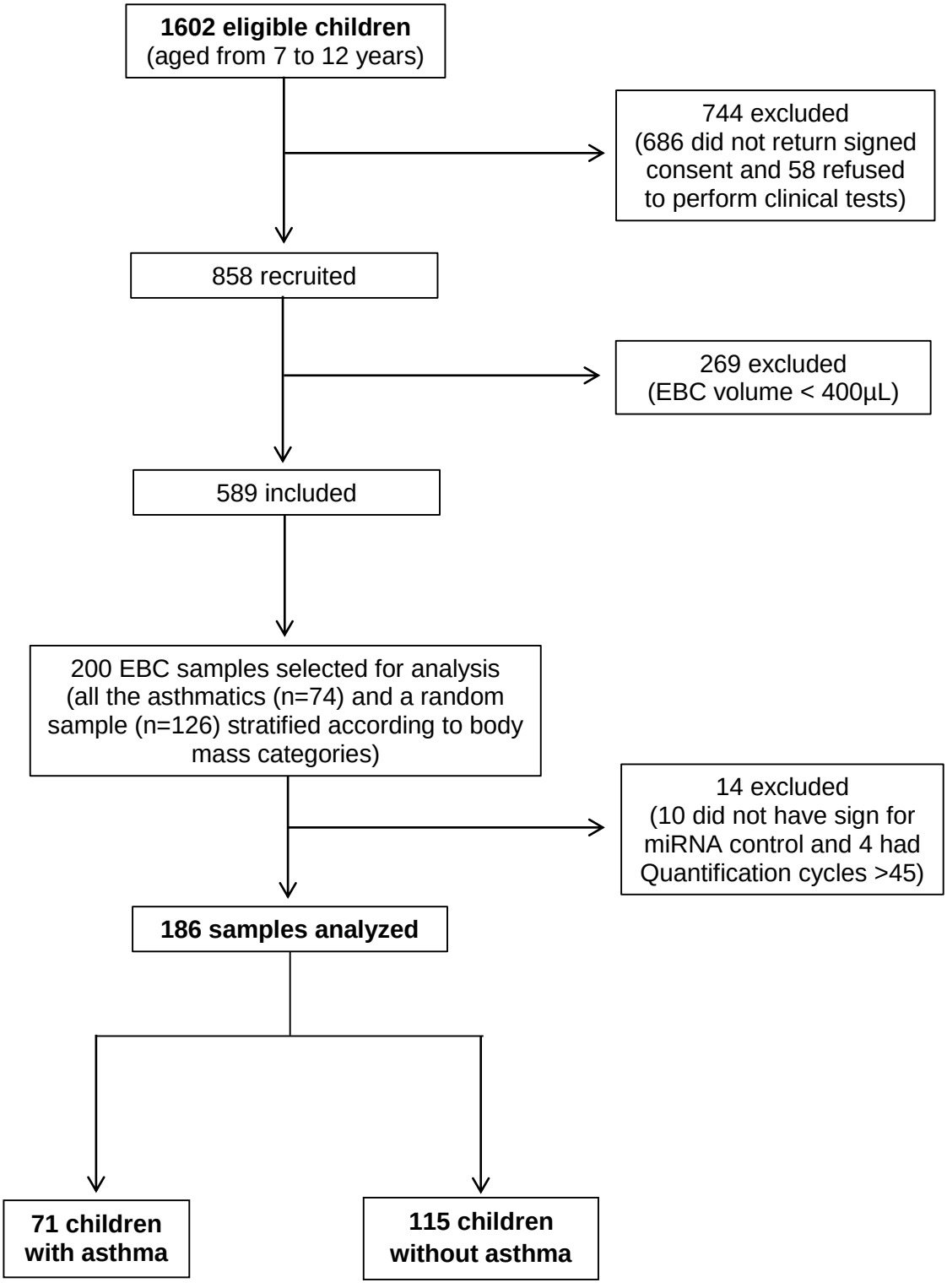


Figure 4. Flow chart of the participants included for EBC miRNAs analysis. Children with asthma were defined based on medical diagnosis or airway reversibility to salbutamol with reported symptoms (wheezing, dyspnea or dry cough) in the past 12 months. Controls were stratified for body mass categories, based on a case: control ratio of 1:2.

Table 2. Characterization of the participants

	Children with asthma[a], n=71	Children without asthma, n=115	*p*-value	Total, n= 186
Age (years)	8.70 0.79	8.73 0.87	0.37	8.71 0.84
Female, n (%)	42 (59.2)	53 (46.1)	0.08	95 (51.1)
Atopic, n (%)[b]	32 (45.1)	40 (34.8)	0.16	72 (38.7)
Body mass categories, n (%)[c]			**<0.01**	
Underweight	3 (4.20)	8 (7.00)		11 (5.90)
Normal weight	42 (59.2)	32 (27.8)		74 (39.8)
Overweight	19 (26.8)	33 (28.7)		52 (28.0)
Obese	7 (9.90)	42 (36.5)		49 (26.3)
Current symptoms[d]				
Breathing difficulties, n (%)	28 (39.4)	7 (6.09)	**<0.01**	35 (18.8)
Irritative cough, n (%)	33 (46.5)	34 (29.6)	**0.02**	67 (36.0)
Lung function				
FVC, L	1.88 (1.64; 206)	2.02 (1.79; 2.18)	0.11	1.96 (1.71; 2.12)
FVC, % predicted	109 (102; 126)	105 (95.3; 113)	**0.01**	108 (98.3; 118)
FEV1, L	1.91 (1.67; 20.8)	1.91 (1.73; 2.13)	**<0.01**	1.80 (1.59; 1.96)
FEV1, % predicted	109 (100; 122)	99.6 (91.2; 109)	**<0.01**	103 (93.9; 112)
FEF25-75, L/s	2.13 (1.65; 2.62)	2.44 (2.01; 2.82)	**0.01**	2.29 (1.84; 2.75)
FEF25-75, % predicted	107 (84.9; 131)	91.0 (79.9; 108)	**<0.01**	97.9 (81.5; 116)
FEV1 reversibility, %	12.7 (3.54; 17.2)	3.75 (0.51; 7.03)	**<0.01**	5.52 (1.87; 12.2)
FEF25-75 reversibility, %	22.5 (6.71; 36.6)	11.4 (2.46; 15.7)	**<0.01**	13.7 (3.17; 23.2)

Exhaled NO, ppb	16.0 (7.00; 36.0)	11.4 (2.46; 15.7)	0.07	12,0 (6,00; 21)
MicroRNA				
miR-21-5p	2.47 (0.59; 4.34)	2.55 (1.20; 6.15)	1.00	2.51 (0.97; 5.50)
miR-126-3p	0.06 ($1.37x10^{-4}$; 0.48)	0.04 ($1.37x10^{-4}$; 0.26)	1.00	0.05 ($1.37x10^{-4}$; 0.29)
miR-133a-3p	0.04 ($6.85x10^{-5}$; 0.43)	0.01 ($6.85x10^{-5}$; 0.29)	0.13	0.02 ($6.85x10^{-5}$; 0.35)
miR-145-5p	0.49 (0.20; 1.30)	0.59 (0.24; 1.25)	0.23	0.55 (0.22; 1.25)
miR-146a-5p	$6.65x10^{-4}$ ($6.65x10^{-4}$; 0.03)	$6.65x10^{-4}$ ($6.65x10^{-4}$; 0.04)	0.70	$6.65x10^{-4}$ ($6.65x10^{-5}$; 0.03)
miR-155-5p	$1.19x10^{-4}$ ($1.19x10^{-4}$; $1.19x10^{-4}$)	$1.19x10^{-4}$ ($1.19x10^{-4}$; 0.01)	0.06	$1.19x10^{-4}$ ($1.19x10^{-4}$; $2.37x10^{-4}$)
miR-221-3p	0.08 ($1.89x10^{-3}$; 0.37)	0.08 ($1.89x10^{-3}$; 0.28)	1.00	0.08 ($1.89x10^{-3}$; 0.29)
miR-328-3p	0.48 (0.02; 1.16)	0.49 (0.15;1.18)	1.00	0.48 (0.12; 1.16)
miR-423-3p	$1.07x10^{-3}$ ($1.07x10^{-3}$; 0.03)	$1.07x10^{-3}$ ($1.07x10^{-3}$; 0.09)	0.21	$1.07x10^{-3}$ ($1.07x10^{-3}$; 0.07)

Data are expressed as medians (25th-75th percentile) unless otherwise stated.

[a]: asthma was defined based on positive bronchodilation or self-reported medical diagnosis with reported symptoms in the past 12 months

[b]: atopy defined by a positive skin prick test to at least one of the allergens;

[c]: according to US Centers for Disease Control

[d]: symptoms in the previous 3 months

FVC: forced vital capacity;

FEV1: forced expiratory volume in the first second;

FEF25-75: forced expiratory flow middle portion of FVC

FEV1 reversibility: forced expiratory volume in the first second after bronchodilation;

FEF25-75 reversibility: forced expiratory flow middle portion of FVC after bronchodilation

Significant differences in **bold**

Table 3. Phenotypic characteristics of children with asthma.

	Asthma[a] phenotypes							
	Children with asthma (n=71)							
	Allergic asthma	**Eosinophilic asthma**	**Obese asthma**	**Treated asthma**	**Symptomatic asthma**	**BD+S+**	**BD+S-**	**BD-S+**
No. of participants	32	19	26	40	43	10	28	33
Age (years)	8.47 (0.57)	9.00 (0.75)	8.63 (0.65)	8.62 (0.75)	8.62 (0.75)	9.10 (0.88)	8.74 (0.86)	8.53 (0.67)
Female, n (%)	17 (53.1)	8 (42.1)	16 (61.5)	21 (52.5)	24 (55.8)	6 (60.0)	18 (64.3)	18 (54.5)
Atopic, n (%)[b]	--	11 (57.9)	10 (38.5)	21 (52.5)	21 (48.8)	4 (40.0)	11 (39.3)	17 (0.52)
Body mass categories, n (%)								
Underweight	2 (6.25)	1 (5.26)	**0 (0.00)****	3 (7.50)	2 (4.65)	1 (10.0)	1 (3.57)	1 (3.03)
Normal weight	20 (62.5)	15 (78.9)	**0 (0.00)****	22 (55.0)	24 (55.8)	5 (50.0)	18 (64.3)	19 (0.58)
Overweight	8 (25.0)	2 (10.5)	**19 (73.1)****	11 (27.5)	13 (30.2)	3 (30.0)	6 (21.4)	10 (30.3)
Obese	2 (6.25)	1 (5.26)	**7 (26.9)****	4 (10.0)	4 (9.30)	1 (10.0)	3 (10.7)	3 (9.09)
Current symptoms[d]								
Breathing difficulties, n (%)	15 (46.9)	**12 (63.2)***	14 (53.8)	**23 (57.5)****	**25 (58.1)****	4 (40.0)	**3 (10.7)****	**21 (63.6)****
Irritative cough, n (%)	15 (46.9)	9 (47.4)	15 (57.7)	**27 (67.5)****	**29 (67.4)****	4 (40.0)	**4 (14.3)****	**25 (75.8)****
Lung function								
FVC, L	1.87 (1.65; 2.05)	2.16 (1.74; 2.60)	2.00 (1.74; 2.08)	1.90 (1.67; 2.05)	1.87 (1.62; 2.05)	1.70 (1.50; 1.87)	1.94 (1.67; 2.10)	1.91 (1.67; 2.06)
FVC, % predicted	106 (102; 126)	109 (99.3; 119)	109 (102; 117)	108 (99.2; 121)	110 (102; 126)	122 (110; 149)	108 (101; 131)	108 (101; 121)

FEV_1, L	1.65 (1.55; 1.95)	1.82 (1.57; 1.98)	1.82 (1.59; 1.93)	1.72 (1.55; 1.93)	1.69 (1.45; 1.93)	1.64 (1.28; 1.71)	1.67 (1.52; 1.84)	1.82 (1.59; 1.99)
FEV1, % predicted	103 (97.8; 120)	103 (96.8; 119)	0.70 (98.7; 112)	102 (96.5; 117)	108 (98.7; 126)	123 (103; 138)	110 (101; 121)	105 (96.9; 117)
FEF25-75, L/s	2.26 (1.74; 2.70)	1.99 (1.72; 2.26)	2.22 (1.73; 2.62)	2.26 (1.83; 2.74)	2.22 (1.74; 2.74)	2.04 (1.61; 2.87)	1.90 (1.62; 2.54)	2.22 (1.84;2.70)
FEF25-75, % predicted	101 (82.5; 126)	107 (90.7; 131)	101 (84.9; 128)	94.7 (81.2; 121)	97.9 (81.9; 122)	103 (78.9; 131)	126 (92.9; 142)	97.9 (82.2; 115)
FEV1 reversibility, %	12.3 (3.89; 16.9)	11.5 (3.09; 13.6)	12.2 (2.63; 15.4)	**4.15 (2.29; 11.7)****	**5.56 (2.29; 12.3)****	18.2 (12.9; 20.2)	**16.3 (13.9; 19.7)****	**2.67 (1.54; 6.54)****
FEF25-75 reversibility, %	16.4 (2.21; 34.6)	23.1 (2.78; 28.5)	16.6 (5.26; 32.3)	**16.4 (2.78; 27.0)***	**15.9 (2.78; 27.0)****	28.5 (8.95; 43.7)	**33.2 (17.8; 44.9)****	**14.4 (2.78; 22.7)****
Exhaled NO (ppb)	**22.0 (11.0; 44.0)***	**51.0 (40.0; 76.0)****	13.0 (6.00; 21.0)	**25.0 (11.5; 45.0)***	18.0 (6.00; 42.0)	7.50 (4.00; 37.0)	12.5 (9.50; 20.5)	24.0 (11.0; 48.0)
MicroRNA								
miR-21-5p	2.77 (0.50; 4.05)	1.80 (0.56; 3.80)	2.33 (0.44; 3.91)	2.47 (0.62; 4.20)	2.20 (0.56; 3.81)	1.58 (0.56; 2.88)	3.40 (0.80; 6.97)	2.46 (0.59; 3.81)
miR-126-3p	0.01 (1.37x10^{-4}; 0.31)	7.85x10-3 (1.37x10^{-4}; 0.24)	0.17 (1.37x10^{-4}; 1.34)	0.03 (1.37x10^{-4}; 0.17)	0.03 (1.37x10^{-4}; 0.20)	6.89x10-3 (1.37x10^{-4}; 0.27)	0.24 (1.37x10^{-4}; 1.19)	0.03 (1.37x10^{-4}; 0.15)
miR-133a-3p	0.03 (6.85x10^{-5}; 0.40)	0.02 (6.85x10^{-5}; 0.38)	0.07 (6.85x10^{-5}; 0.37)	0.04 (6.85x10^{-5}; 0.41)	0.02 (6.85x10^{-5}; 0.34)	4.42sx10^{-3} (6.85x10^{-5}; 0.07)	0.20 (6.85x10^{-5}; 0.83)	0.03 (6.85x10^{-5}; 0.38)
miR-145-5p	0.48 (0.24; 1.32)	0.36 (0.11; 0.84)	0.55 (0.25; 1.18)	0.54 (0.22; 1.47)	0.44 (0.15; 1.25)	0.33 (0.13; 0.54)	0.55 (0.27; 1.64)	0.49 (0.20; 1.33)
miR-146a-5p	6.65x10^{-4} (6.65x10^{-4}; 2.37x10^{-3})	6.65x10^{-4} (6.65x10^{-4}; 0.05)	6.65x10^{-4} (6.65x10^{-4}; 0.03)	6.65x10^{-4} (0.02; 0.02)	6.65x10^{-4} (6.65x10^{-4}; 0.02)	0.01 (6.65x10^{-4}; 0.07)	6.65x10^{-4} (6.65x10^{-4}; 0.04)	6.65x10^{-4} (6.65x10^{-4}; 4.66x10^{-3})
miR-155-5p	1.19x10^{-4} (1.19x10^{-4}; 1.19x10^{-4})	1.19x10^{-4} (1.19x10^{-4}; 1.19x10^{-4})	1.19x10^{-4} (1.19x10^{-4}; 1.19x10^{-4})	1.19x10^{-4} (1.19x10^{-4}; 1.19x10^{-4})	1.19x10^{-4} (1.19x10^{-4}; 1.19x10^{-4})	1.19x10^{-4} (1.19x10^{-4}; 1.19x10^{-4})	1.19x10^{-4} (1.19x10^{-4}; 1.19x10^{-4})	1.19x10^{-4} (1.19x10^{-4}; 1.19x10^{-4})
miR-221-3p	0.05 (1.89x10^{-3}; 0.32)	0.06 (1.89x10^{-3}; 0.46)	0.09 (1.89x10^{-3}; 0.44)	0.10 (1.89x10^{-3}; 0.45)	0.06 (1.89x10^{-3}; 0.36)	0.07 (1.89x10^{-3}; 0.36)	0.12 (1.89x10^{-3}; 0.42)	0.06 (1.89x10^{-3}; 0.31)
miR-328-3p	0.73 (0.26; 1.27)	0.42 (0.03; 0.90)	0.26 (5.98x10^{-3}; 0.98)	0.51 (0.09; 1.33)	0.48 (0.08; 1.10)	0.25 (5.98x10^{-3}; 0.63)	0.43 (5.98x10^{-3}; 1.17)	0.55 (0.10; 1.48)

miR-423-3p	$1.07x10^{-3}$ ($1.07x10^{-3}$; 0.02)	$1.07x10^{-3}$ ($1.07x10^{-3}$; 0.04)	$1.07x10^{-3}$ ($1.07x10^{-3}$; $1.07x10^{-3}$)	$1.07x10^{-3}$ ($1.07x10^{-3}$; $9.88x10^{-3}$)	$1.07x10^{-3}$ ($1.07x10^{-3}$; 0.02)	$1.07x10^{-3}$ ($1.07x10^{-3}$; 0.11)	$1.07x10^{-3}$ ($1.07x10^{-3}$; 0.04)	$1.07x10^{-3}$ ($1.07x10^{-3}$; $2.14x10^{-3}$)

Data are expressed as medians (25th-75th percentile) unless otherwise stated.

[a]: asthma was defined based on positive bronchodilation or self-reported medical diagnosis with reported symptoms in the past 12

[b]: atopy defined by a positive skin prick test to at least one of the allergens;

[c]: according to US Centers for Disease Control

[d]: symptoms in the previous 3 months

FVC: forced vital capacity;

FEV1: forced expiratory volume in the first second;

FEF25-75: forced expiratory flow middle portion of FVC

FEV1 reversibility: forced expiratory volume in the first second after bronchodilation;

FEF25-75 reversibility: forced expiratory flow middle portion of FVC after bronchodilation

Allergic asthma: defined by “asthma” in a child with positive skin prick test

Eosinophilic asthma: defined by “asthma” in a child with exhaled nitric oxide above 35 ppb

Obese asthma: defined by “asthma” in an overweight or obese child

Treated asthma: defined by “asthma” in a child currently using anti-asthma medication

Symptomatic asthma: defined by “asthma” in a child with current symptoms

BD+S+: Positive bronchodilation with asthma symptoms defined by “asthma” in a child with a current positive bronchodilation test and symptoms

BD+S-: Positive bronchodilation without asthma symptoms defined by “asthma” in a child with a current positive bronchodilation test and without symptoms

BD-S+: Negative bronchodilation with asthma symptoms defined by “asthma” in a child with a current negative bronchodilation test and with symptoms

Significant differences **in bold**

*: *p*-value <0.05

**: *p*-value <0.01

***:*p*-value<0.001

3.2. Questionnaire

A self-administered ISAAC-based questionnaire, which included questions regarding social, demographic and behavioral characteristics, questions regarding respiratory/allergic health and current symptoms (previous 3 months) was filled by children caregivers and reviewed by a research nurse. Wheezing symptoms were defined by a positive answer to the question “Did your child have wheezing or whistling in the chest, in the past 12 months?”. Cough symptoms were defined by a positive answer for any of the two following questions “Did your child suffer coughing at night in the last 12 months?” or “Did your child suffer coughing more than three months in the last year?”. Children were considered to have current symptoms in the previous 3 months if there was a positive answer to the question “During the past 3 months, has your child had any of the following symptoms?”: “Irritative cough” and “Breathing difficulties”.

3.3. Assessments

Data on anthropometry (weight and height) was used to calculate body mass index (BMI). Weight and percentage of body fat were measured using a digital scale (Tanita™ BC-418 Segmental Body Analyzer) and height was measured with a portable stadiometer and was recorded in kilograms (Kg), Kg per square meters (kg/m^2) and centimeters (cm), respectively. BMI was classified according to age- and sex-specific percentiles defined by the US Centers for Disease Control and Prevention (CDC) (Kuczmarski, Ogden, Grummer-Strawn et al., 2000).

Lung function and airway reversibility were assessed by spirometry according to the official ATS/ERS guidelines (Miller, Hankinson, Brusasco et al., 2005) and were recorded before and 15 minutes after inhalation of 400µg of salbutamol.

Levels of exhaled nitric oxide (NO) were measured to assess airway inflammation using the NObreath analyzer (Bedfont Scientific Ltd., Rochester, Kent, UK). The results were expressed as parts per billion (ppb) and stratified according to the official ATS guidelines for children (Dweik, Boggs, Erzurum et al., 2011).

Skin-prick-tests (SPT) were performed on children forearm to evaluate allergic sensitization using a QuickTest™ (Panatrex Inc., Placentia, California, USA applicator) which contained house dust mite, mix of weeds (*Urtica dioica*, *Plantago lanceolate* and *Artemisia vulgaris*), mix of grasses (*Agrostis stolonifera*, *Anthoxathum odoratum*, *Dactylis glomerata, Lolium perenne*, *Arrhenatherium elatius*, *Festuca rubra*, *Poa pratensis*, *Holcus lanatus*, *Phleum pratense*, *Secale cereal*), cat dander, dog dander and *Alternaria alternata*, negative control (extracts dilutant), and a positive control consisting of histamine at 10mg/ml (Hall Allergy, Netherlands). Results were read 15 minutes afterwards and atopy was defined by a positive SPT to at least one of the allergens.

3.4. Exhaled breath condensate (EBC) collection and miRNA quantification

Exhaled breath condensate was collected from children by breathing (regular tidal volumes and respiratory rate) 10 to 15 minutes to an exhaled air condensing system (portable Turbo DECCS) (**Figure 5**) (Horvath, Hunt, Barnes et al., 2005). Exhaled breath condensate samples were transferred to sterile tubes and stored at -80ºC until laboratory analysis.

Based on previous studies, a panel of eleven specific miRNAs was chosen for analysis: let7a-5p; miR21-5p; miR126-3p; miR133a-3p; miR145-5p; miR146a-5p; miR155-5p; miR221-3p; miR328-3p; miR-423-3p and miR-1248 (F. Liu, Qin, Xu et al., 2012; T. X. Lu, Sherrill, Wen et al., 2012; Qin, Xu, Mei et al., 2012; Pinkerton, Chinchilli, Banta et al., 2013; Suojalehto, Lindstrom, Majuri et al., 2014; Wu, Wang, Zhu et al., 2014; Herbert, Sebesfi, Zeng et al., 2015; Kho, Sharma, Davis et al., 2016; Wang, Huang, Liang et al., 2016; Lacedonia, Palladino, Foschino-Barbaro et al., 2017; Malmhall, Johansson, Winkler et al., 2017; Trinh, Pham, Kim et al., 2017). MicroRNA 423-3p was included for normalization purposes but expression turned out to be too variable for the purpose.

Total RNA was extracted by guanidinium thiocyanate-phenol-chloroform extraction from 0.5 ml of EBC samples, which had been stored at -80 ˚C in flat bottom tubes

immediately after collection. Prior to RNA extraction, the EBC samples were spiked with synthetic UniSp6 RNA (Exiqon) for extraction control purposes. The extracted RNA was quality controlled with a Bioanalyzer (Agilent Technologies, USA) using the Small RNA Kit (Agilent) according to manufacturer's instructions. (**Figure 6**)

Reverse transcription (RT) and real-time quantitative polymerase chain reaction (qPCR) were carried out with dedicated reagents from Exiqon (Qiagen USA), employing Locked Nucleic Acid (LNA™) technology. LNAs are a class of high-affinity RNA analogs in which the ribose ring is "locked" in the ideal conformation for Watson-Crick binding. This results in unprecedented sensitivity and specificity and makes LNA oligonucleotides ideal for the detection of small or highly similar RNA targets. RNA extracted from each EBC sample was reverse transcribed in duplicate with the Universal cDNA synthesis kit II (Exiqon). Ten microliters of RNA solution were used per RT reaction. The resulting cDNA was diluted 10x and quantified by qPCR in 10 µl reactions using miRNA-specific LNA oligos and ExiLENT SYBR Green master mix (Exiqon) (**Table 4**)

Quantitative PCR was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems) with the following cycling conditions: denaturing at 95 ºC for 10 sec, annealing and extension at 60 ºC for 1 min (45 cycles). All assays were done in duplicate; one no-template and one positive control (UniSp6) were used in each experiment. For inter-plate calibration, one specific sample was amplified for UniSp6 in duplicate, in the same wells of each individual plate (**Table 5**).

GenEX software (MultiD Analyses AB, Göteborg, Sweden) was used to analyze and normalize the miRNA-qPCR data. This program allows the correction of PCR efficiencies, the compensation for differences between runs by normalizing with interplate calibrators, and normalization with miRNA let7a-5p, chosen by NormFinder and geNorm, which looked at gene expression variance to choose the most stably expressed genes. All samples with a Quantification cycles (Cq) lower than 45 were included.

Figure 5. Collection of EBC. To collect exhaled breath condensate, children breathe normally into a mouth piece connected during 10 to 15 minutes to an exhaled air condensing system (portable Turbo DECCS).

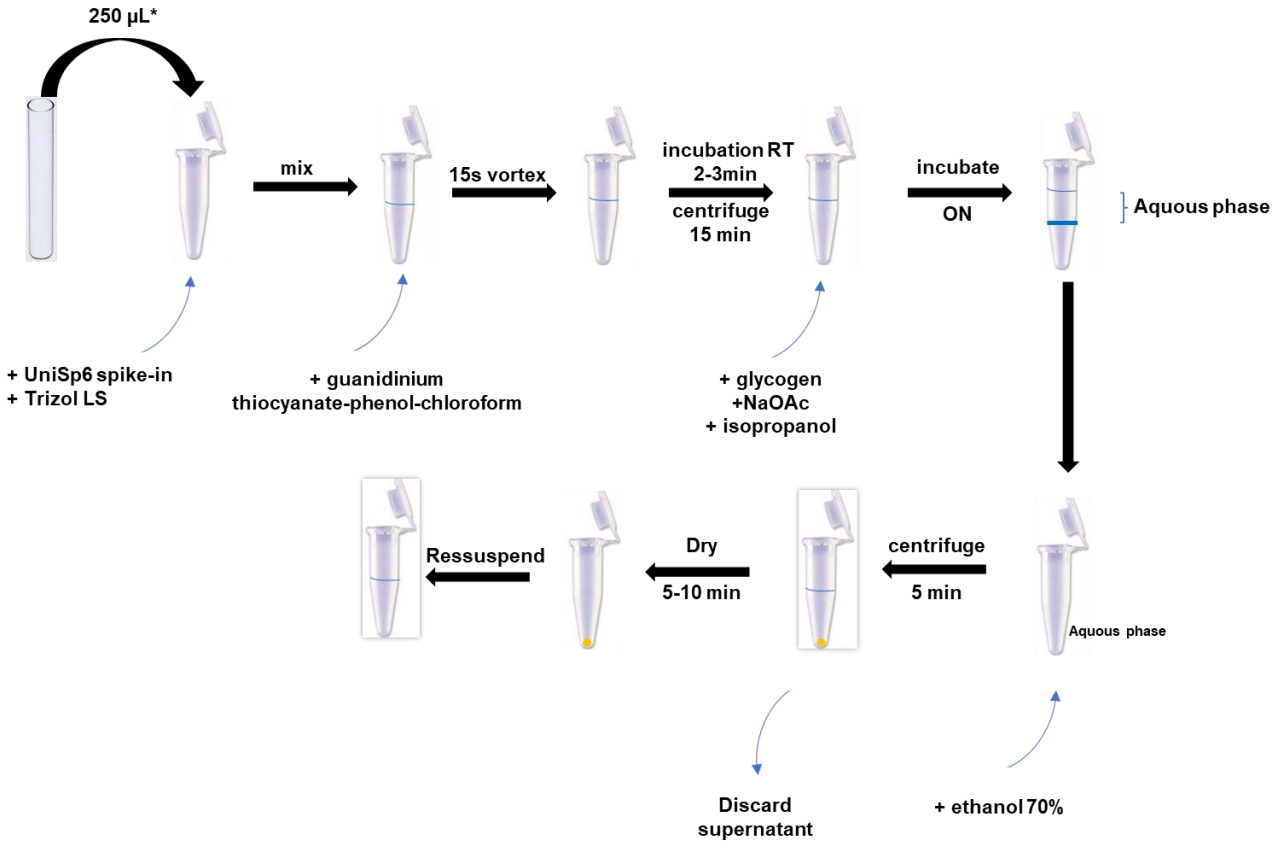


Figure 6. Extraction of microRNAs from exhaled breath condensate. * In samples with lower volumes, MilliQ water was added until it had reached 250 µL. s: seconds; mi: minutes; LS: liquid samples; NaOAc: sodium acetate; ON: overnight

Table 4. Reaction of first-strand cDNA synthesis.

Reagents	Volume per sample
Exiqon 5x reaction buffer	6 µL
Nuclease-free water	11 µL
Exiqon enzyme mix	3 µL
Template RNA	10 µL

Table 5. Conditions of Reverse transcription and real-time quantitative polymerase chain reaction

		PCR (45 cycles)	
	Denature	**Anneal**	**Extend**
Temperature (ºC)	95	60	60
Time	10s	1min	1min

3.5. Statistical Analysis

The normality distribution of continuous variables was tested by the Kolmogorov–Smirnov test. The Mann–Whitney test was used for inferential analysis when non-Gaussians distributions were observed. The categorical data were compared using the chi-squared test. Significant differences were reported with an α-value inferior to 5% ($p<0.05$).

To identify miRNA patterns, principal component analysis (PCA) is one of the multivariate statistical techniques applicable. We exclude miR-1248 of the analysis since its expression turned out to be missed in more than 80% of the samples. Principal component analysis was used to examine the correlational structure of the data based on 9 miRNAs. Kaiser normalization was performed, and adequacy values were above 0.6, value required for a good factor analysis. Only components with eigenvalues above 1.0 were retained in the solution. Factor loadings in the factorial analysis can be interpreted as correlation coefficients between miRNAs and those above 0.5 were

considered as significantly contributing to a cluster. Principal component analysis exhibited two factors that satisfactorily described the distributions of microRNAs levels in children. The two principal components (PC) explained 53.5% of the variance, and positive loadings in each pattern were as follows: PC 1 (32.0% of variance) showed high positive loadings of miR-126-3p, miR-133a-3, miR-145-5p, miR-221-3p and miR-328-3p and PC 2 (21.5% of variance) showed high positive loadings of miR-146a-5p and miR-423-3p (**Figure 7**).

Linear and logistic regression models were performed to determine the association between miRNAs as an individual and as a group and asthma, asthma phenotypes, exhaled NO, lung function and current symptoms defined by irritative cough and breathing difficulties in the previous 3 months. Age, sex, exhaled NO, atopy, body mass categories, anti-asthma medication, positive bronchodilation, reported symptoms in the past 12 months and asthma were analyzed as potential confounders. A 0.05 level of significance and 95 % CI were considered.

The SPSS statistical package software v25.0 (IBM, USA) was used.

Figure 7. Principal component analysis (PCA). Principal Component (PC) 1 showed high positive loadings of miR-126-3p, miR-133a-3p, miR-145-5p, miR-221-3p and miR-328-3p and PC 2 showed high positive loadings of miR-146a-5p and miR-423-3p. Only factor loadings above 0.5 were considered as significantly contributing to a cluster.
miR: microRNA

4. Results

Positive and significant associations were found between miR-126-3p and asthma (β=1.65, 95% CI= 1.09; 2.49) (**Figure 8a, table 6**) and between miR-133a-3p and positive bronchodilation without symptoms (β=2.53, 95% CI= 1.02; 6.24) (**Figure 8b, table 6**). Principal component 1 typified by miR-126-3p, miR-133a-3, miR-145-5p, miR-221-3p and miR-328-3p was also positively and significantly associated with asthma (β=1.60, 95% CI= 1.00; 2.54) (**Figure 8c, table 6**).

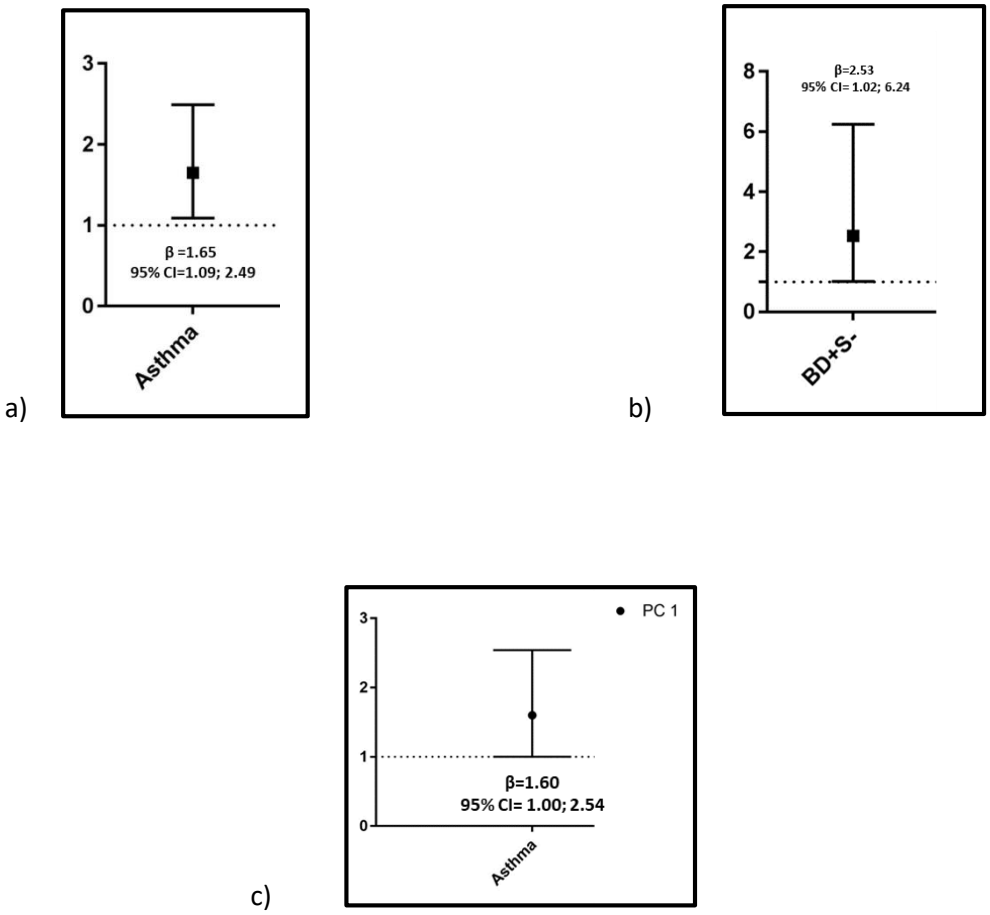


Figure 8. Association between miRNAs and PCs and asthma and asthma phenotypes. a) miR-126-3p and asthma; b) miR-133a-3p and positive bronchodilation without symptoms (BD+S-); c) PC1 and asthma. Associations were adjusted for age, sex, exhaled NO, atopy, body mass categories according to CDC and currently using anti-asthma medication. PC1: Principal component 1 typified by miR-126-3p, miR-133a-3p, miR-145-5p, miR-221-3p and miR-328-3p

Table 6. Associations between miRNAs and asthma and asthma phenotypes

	Asthma[a]		Allergic Asthma		Eosinophilic Asthma		Obese asthma		Treated Asthma	
	Confounder adjusted[b]		Confounder adjusted[c]		Confounder adjusted[d]		Confounder adjusted[e]		Confounder adjusted[f]	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI
miR-126-3p	**1.65**	**1.09; 2.49**	0.89	0.63; 1.24	0.76	0.43; 1.35	1.06	0.81; 1.39	0.90	0.67; 1.22
miR-133a-3p	1.40	0.80; 2.46	0.77	0.36; 0.87	0.39	0.10; 1.56	0.92	0.48; 1.76	1.31	0.68; 2.52
miR-145-5p	1.12	0.96; 1.32	0.82	0.59; 0.80	0.64	0.32; 1.30	0.97	0.81; 1.16	0.95	0.77; 1.15
miR-146a-5p	6.97	0.32; 154	0.97	0.23; 4.16	0.99	0.12; 8.17	1.04	0.24; 4.49	0.74	0.17; 3.27
miR-221-3p	1.36	0.84; 2.22	0.73	0.33; 1.62	0.52	0.15; 1.80	0.99	0.71; 1.39	1.06	0.76; 1.47
miR-328-3p	1.02	0.85; 1.24	1.01	0.77; 1.32	0.60	0.18; 1.32	0.94	0.75; 1.19	1.22	0.92; 1.60
miR-423-3p	1.85	0.75; 4.58	1.91	0.32; 11.5	0.80	0.16; 3.98	0.78	0.29; 2.11	0.31	0.01; 8.75
PC 1	**1.60**	**1.00; 2.54**	0.87	0.57; 1.33	0.42	0.09; 1.88	0.96	0.68; 1.36	0.99	0.70; 1.40
PC 2	1.18	0.85; 1.63	1.15	0.77; 1.74	1.13	0.76; 1.67	1.01	0.73; 1.40	0.84	0.58; 1.21

Table 6. Associations between miRNAs and asthma and asthma phenotypes

	Symptomatic Asthma		BD+S+		BD+S-		BD-S+	
	Confounder adjusted[b]		Confounder adjusted[b]		Confounder adjusted[b]		Confounder adjusted[b]	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI
miR-126-3p	0.77	0.49; 1.20	0.31	0.04; 2.57	1.30	0.83; 2.04	1.14	0.71; 1.84
miR-133a-3p	0.34	0.16; 1.02	0.13	$5.00x10^{-3}$; 2.93	**2.53**	**1.02; 6.24**	0.88	0.36; 2.15
miR-145-5p	0.63	0.36; 1.12	0.41	0.09; 1.83	1.58	0.90; 2.79	0.94	0.60; 1.47
miR-146a-5p	0.73	0.06; 9.26	0.60	0.03; 12.1	1.38	0.11; 17.5	0.77	0.02; 36.9
miR-221-3p	0.64	0.33; 1.23	0.53	0.10; 2.74	1.56	0.81; 3.02	0.96	0.51; 1.81
miR-328-3p	0.93	0.67; 1.30	0.36	0.09; 1.54	1.07	0.77; 1.50	1.26	0.79; 2.01
miR-423-3p	0.51	0.02; 11.6	0.54	0.02; 11.7	1.95	0.09; 44.1	0.84	0.03; 24.3
PC 1	0.61	0.33; 1.12	0.10	0.01; 1.53	1.65	0.90; 3.02	1.06	0.59: 1.91
PC 2	1.10	0.75; 1.63	1.05	0.70; 1.56	0.91	0.61; 1.34	1.07	0.52; 2.20

[a]: asthma was defined based on positive bronchodilation or self-reported medical diagnosis with reported symptoms in the past 12 months

[b]: Logistic regression adjusted for: age, sex, exhaled NO, atopy, body mass categories according to CDC and currently using anti-asthma medication

[c]: Logistic regression adjusted for: age, sex, exhaled NO, body mass categories according to CDC and currently using anti-asthma medication

[d]: Logistic regression adjusted for: age, sex, atopy, body mass categories according to CDC and currently using anti-asthma medication

[e]: Logistic regression adjusted for: age, sex, exhaled NO, atopy and currently using anti-asthma medication

[f]: Logistic regression adjusted for: age, sex, exhaled NO, atopy and body mass categories according to CDC

PC 1: Principal component 1 typified by miR-126-3p, miR-133a-3, miR-145-5p, miR-221-3p and miR-328-3p

PC 2: Principal component 2 typified by miR-146a-5p and miR-423-3p

Allergic asthma: defined by “asthma” in a child with positive skin prick test

Eosinophilic asthma: defined by “asthma” in a child with exhaled nitric oxide above 35 ppb

Obese asthma: defined by “asthma” in an overweight or obese child

Treated asthma: defined by “asthma” in a child currently using anti-asthma medication

Symptomatic asthma: defined by “asthma” in a child with current symptoms

BD+S+: Positive bronchodilation with asthma symptoms defined by “asthma” in a child with a current positive bronchodilation test and symptoms

BD+S-: Positive bronchodilation without asthma symptoms defined by “asthma” in a child with a current positive bronchodilation test and without symptoms

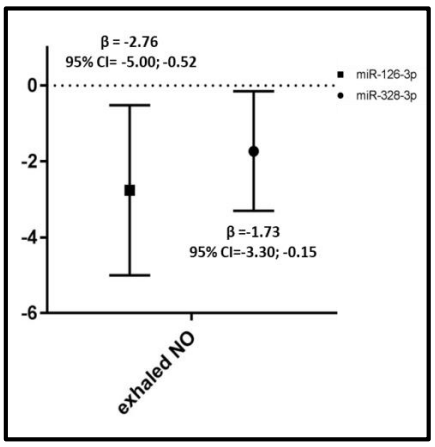
BD-S+: Negative bronchodilation with asthma symptoms defined by “asthma” in a child with a current negative bronchodilation test and with symptoms

Significant differences in **bold**

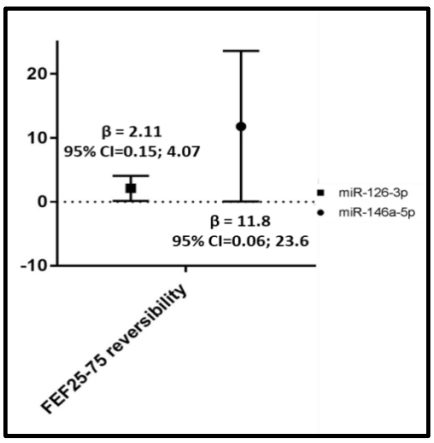
Higher levels of miR-126-3p and miR-328-3p were associated with lower eNO (β=-2.76, 95% CI= -5.00; -0.52 and β=-1.73, 95% CI= -3.30; -0.15, respectively) (**Figure 9a, table 7**). Further, miR-126-3p and miR-146a-5p were associated with higher small airways salbutamol response after bronchodilation (β=2.11, 95% CI= 0.15; 4.07 and β=11.8, 95% CI= 0.06; 23.6, respectively) (**Figure 9b, table 7**). Also, miR-328-3p was negatively associated with FEV1 after bronchodilation (β= -0.62, 95% CI= -1.22; -0.03) (**Figure 9c, table 7**). Regarding current symptoms, a positive association was found between miR-221-3p and breathing difficulties (β=0.15, 95% CI= 0.02; 0.97) (**Figure 9d, table 7**).

Principal component 1 and PC 2 were also significantly associated with exhaled NO (β= -3.54, 95% CI= -6.47; -0.62) (**Figure 9e, table 7**) and with small airways salbutamol response after bronchodilation (β= 2.56, 95% CI= 0.07; 5.05) (**Figure 9f, table 7**), respectively.

a)



b)



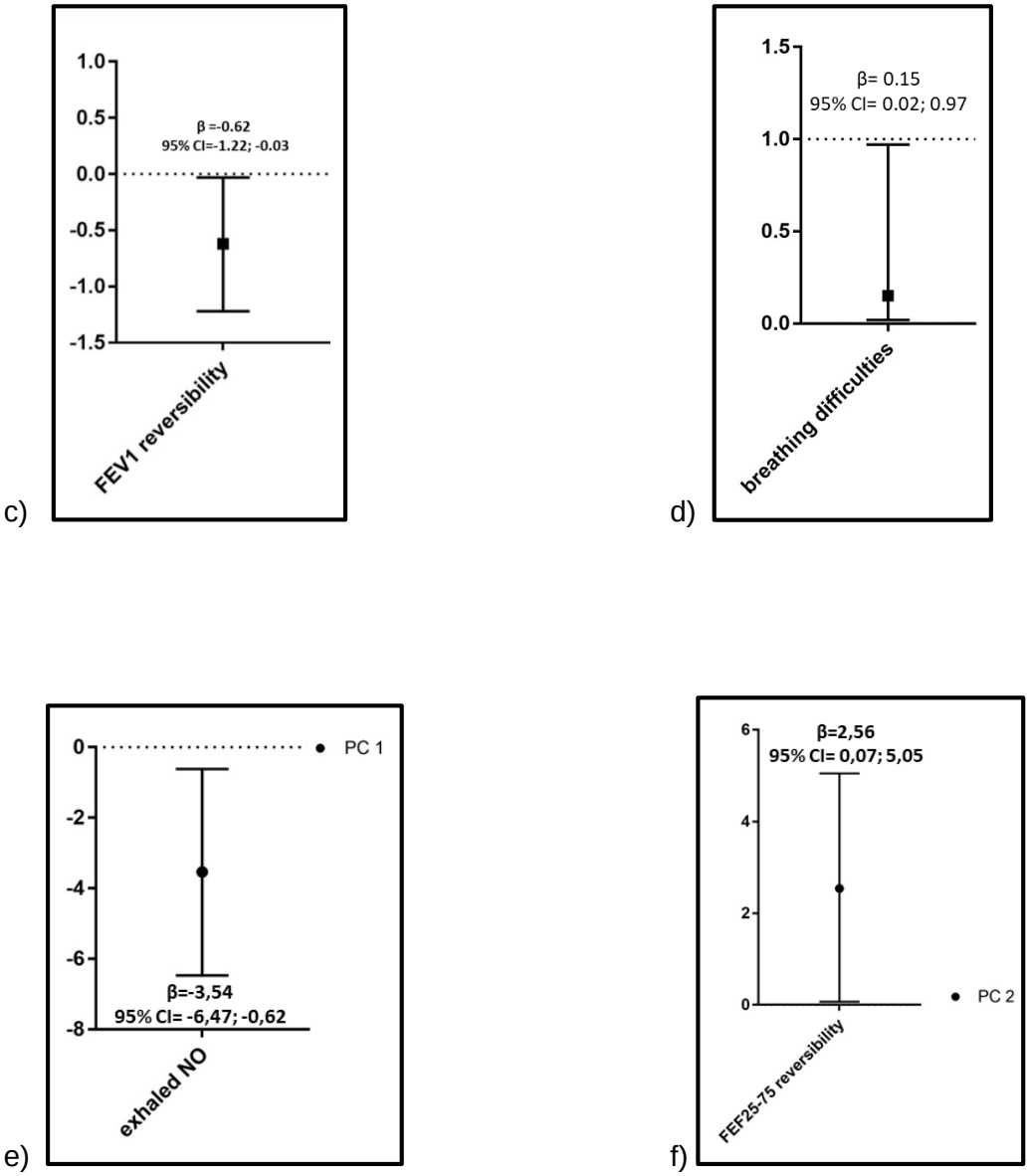


Figure 9. Association between miRNAs and PCs and exhaled nitric oxide (NO), lung function and current symptoms. a) miR-126-3p and miR-328-3p and exhaled NO; b) miR-126-3p and miR-146a-5p and FEF25-75 after bronchodilation; c) miR-328-3p and FEV1 after bronchodilation; d) miR-221-3p and current symptoms; e) PC 1 and exhaled NO; f) PC 2 and FEF 25-75 after bronchodilation. a) and e) were adjusted for age, sex, atopy, body mass categories according to CDC and asthma defined based on positive bronchodilation or self-reported medical diagnosis with reported symptoms in the past 12 months; b), c), d) and f) were adjusted for age, sex, exhaled NO, atopy, body mass categories according to CDC and asthma defined based on positive bronchodilation or self-reported medical diagnosis with reported symptoms in the past 12 months. FEV1: forced expiratory volume in the first second. FEF25-75: forced expiratory flow middle portion of FVC. PC 1: Principal component 1 typified by miR-126-3p, miR-133a-3, miR-145-5p, miR-221-3p and miR-328-3p; PC 2: Principal component 2 typified by miR-146a-5p and miR-423-3p

Table 7. Associations between miRNAs, exhaled NO and lung function and current symptoms

	Exhaled NO		FEV1 (% predicted)		FEF25-75 (% predicted)		FEV1 reversibility		FEF25-75 reversibility	
	Confounder adjusted[a]		Confounder adjusted[b]		Confounder adjusted[b]		Confounder adjusted[b]		Confounder adjusted[b]	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI
miR-126-3p	**-2.76**	**-5.00; -0.52**	-0.25	-1.89; 1.39	2.90	-0.28; 6.08	-0.30	-1.16; 0.56	**2.11**	**0.15; 4.07**
miR-133a-3p	-0.85	-5.44; 3.75	-0.23	-3.49; 3.04	-0.33	-6.74; 6.08	-0.23	-1.88; 1.43	-0.69	-4.51; 3.13
miR-145-5p	-1.15	-2.44; 0.15	0.05	-0.89; 0.98	-0.03	-1.86; 1.80	0.11	-0.38; 0.60	0.42	-0.71; 1.55
miR-146a-5p	-9.35	-23.0; 4.33	0.04	-9.78; 9.86	7.63	-11.6; 26.9	0.16	-5.00; 5.32	**11.8**	**0.06; 23.6**
miR-221-3p	-0.82	-3.74; 2.10	1.14	-0.92; 3.20	-1.60	-5.64; 2.44	0.05	-1.04; 1.13	-0.92	-3.42; 1.58
miR-328-3p	**-1.73**	**-3.30; -0.15**	-0.17	-1.32; 0.97	-0.71	-2.96; 1.54	**-0.62**	**-1.22; -0.03**	-0.78	-2.16; 0.61
miR-423-3p	-6.00	-13.1; 1.06	0.60	-4.47; 5.68	5.47	-4.45; 15.4	-0.24	-2.91; 2.43	6.03	-0.06; 12.1
PC 1	**-3.54**	**-6.47; -0.62**	0.19	-1.94; 2.34	0.76	-3.43; 4.96	-0.35	-1.49; 0.79	1.19	-1.41; 3.79
PC 2	0.43	-4.10; 1.77	-0.11	-2.18; 1.96	2.78	-1.25; 6.82	-0.01	-1.11; 1.10	**2.56**	**0.07; 5.05**

Table 7. Associations between miRNAs, exhaled NO and lung function and current symptoms

	Breathing difficulties		Irritative cough	
	Confounder adjusted[c]		Confounder adjusted[c]	
	β	95% CI	β	95% CI
miR-126-3p	0.67	0.38; 1.20	0.76	0.53; 1.08
miR-133a-3p	0.65	0.31; 1.35	0.67	0.39; 1.17
miR-145-5p	0.58	0.33; 1.03	0.80	0.60; 1.06
miR-146a-5p	1.19	0.28; 5.12	1.05	0.25; 4.37
miR-221-3p	**0.15**	**0.02; 0.97**	0.50	0.22; 1.12
miR-328-3p	0.71	0.46; 1.10	1.04	0.87; 1.23
miR-423-3p	0.53	0.07; 4.01	0.53	0.11; 2.50
PC 1	0.42	0.16; 1.13	0.70	0.44; 1.09
PC 2	1.13	0.81; 1.57	1.09	0.81; 1.47

[a]: Linear regression adjusted for: age, sex, atopy, body mass categories according to CDC and asthma defined based on positive bronchodilation or self-reported medical diagnosis with reported symptoms in the past 12 months

[b]: Linear regression adjusted for: age, sex, exhaled NO, atopy, body mass categories according to CDC and asthma defined based on positive bronchodilation or self-reported medical diagnosis with reported symptoms in the past 12 months

[c]: Logistic regression adjusted for: age, sex, exhaled NO, atopy, body mass categories according to CDC and asthma defined based on positive bronchodilation or self-reported medical diagnosis with reported symptoms in the past 12 months

PC 1: Principal component 1 typified by miR-126-3p, miR-133a-3, miR-145-5p, miR-221-3p and miR-328-3p

PC 2: Principal component 2 typified by miR-146a-5p and miR-423-3p

FEV1: forced expiratory volume in the first second;

FEF25-75: forced expiratory flow middle portion of FVC

FEV1 reversibility: forced expiratory volume in the first second after bronchodilation;

FEF25-75 reversibility: forced expiratory flow middle portion of FVC after bronchodilation

Significant differences in **bold**

5. Discussion

Our study shows that miRNA can be measured in exhaled breath condensate of schoolchildren, being significantly associated with asthma, positive bronchodilation without symptoms, exhaled nitric oxide, airway reversibility and breathing difficulties. We also found significant associations between miRNA profiles assessed by principal component analysis and asthma, exhaled nitric oxide and small airways response to salbutamol.

Our findings have few limitations. First it is a cross-sectional study that limits the evaluation through the time, not allowing to establish causal relationships between miRNAs profile, lung function and asthma diagnosis. Moreover, the use of case-control studies has often been associated with biased estimates of diagnostic accuracy, due to the incorrect sampling of subjects. However, our study was nested within a well-defined cohort of children where the 'true' disease status was obtained for all with the reference standard. Hence, there was no referral or partial verification bias. In fact, we may assume within this design our findings to be similar if the all 1602 participants were included in the analysis. Secondly, there was *a priori* selection of miRNAs based on previous studies. Nevertheless, these miRNAs were chosen based on previous studies which address their association with asthma and obesity. Thirdly, our findings may lack external validity as generalization of our observations may not be valid in different age groups. Further, grouping children with asthma according to different phenotypes may reduce the power to detect differences, considering the sample number. As such, significant associations were only found when all the children with asthma were included.

Our study has also important strengths. Asthma, a heterogeneous condition, was defined based not only on the medical report but also on the presence of symptoms and evidence of airway reversibility. Additionally, and contrarily to previous studies, we used different phenotypes allowing the discrimination between children with asthma reducing the heterogeneity within each group. However, different participants were included in more than one phenotype and had an overlapped association. Also, a detailed health data was collected allowing for a relatively unbiased estimate of the prevalence of asthma, allergy sensitization, respiratory symptoms and body mass categories and a

large number of EBC samples were analyzed. Furthermore, the collection of EBC is simple, safe, non-invasive and highly repeatable. Also, qPCR has become the most widely used method for the study of microRNAs because it is fast, extremely sensitive and offers linear detection over several orders of magnitude and the technology employed is ideal for miRNA profiling from difficult samples such as EBC (Universal, 2011; QIAGEN, 2017). We assessed the effects of miRNAs either alone or as profile, since they can be expressed as a pattern, rather than individual. Accordingly, Panganiban et al. suggested that expression profiling could be a useful tool to phenotype asthma (Panganiban, Wang, Howrylak et al., 2016).

Asthma is an umbrella term to describe a clinical problem, which requires careful multidisciplinary assessment (Pavord, Beasley, Agusti et al., 2018). Further, this disease is heterogeneous and the traditional diagnostic approaches cluster patients according to etiologic diagnosis and treatment. As such, there is a need to distinguish patients not only by their phenotypic characteristics but also by the pathophysiological mechanisms of the disease, in order to offer precision management and targeted treatment. In this context arises the term endotype that implicates the searching for new biomarkers, like miRNAs. In fact, asthma incorporates a variety of underlying endotypes, sharing common symptoms and phenotypic characteristics (Haldar, Pavord, Shaw et al., 2008; Zoratti, Krouse, Babineau et al., 2016). However, it is likely that some mechanisms overlap one or more endotypes. This heterogeneity was also seen in our study where we identified children with positive bronchodilation without symptoms and children with asthma defined by positive bronchodilation or medical diagnosis with reported symptoms in the past 12 months by two different miRNAs. These results reflect that the use of aggregated definitions can contribute to inconsistent findings between studies.

We performed a pre-selection of miRNAs based on previous studies addressing their potential role in asthma and obesity. Since asthma and obesity are characterized by chronic inflammation, it is likely that miRNAs might be deregulated in these diseases, modulating immune cells that may be found in EBC. Further, obesity may be a possible feature of an asthma endotype. Specifically, miR-21, miR-133, miR-155 and miR-328 were previously identified in EBC (Pinkerton, Chinchilli, Banta et al., 2013), miR-126 stimulates Th-2 mediated allergic inflammation (Nejad, Stunden, & Gantier, 2018), miR-145 has been linked to TNF-α production , miR-146 was one of the first miRNAs identified to be influenced in immune cells stimulated by pro-inflammatory cytokines (Rebane & Akdis, 2013), miR-221 is a positive regulator of innate immune responses (Nejad,

Stunden, & Gantier, 2018) and miR-423 was previously identified as increased in obese children (Prats-Puig, Ortega, Mercader et al., 2013) and were therefore chosen.

This study assessed the effect of individual and grouped miRNAs on asthma, asthma phenotypes, lung function and airway reversibility, exhaled NO and current symptoms, while most previous studies have focused predominantly on the differences between miRNAs levels as an individual among participants with asthma (Qin, Xu, Mei et al., 2012; Pinkerton, Chinchilli, Banta et al., 2013; Wu, Wang, Zhu et al., 2014). Furthermore, most of the studies of miRNAs in asthma are quite heterogeneous, both in disease definition and in different matrixes assessed, such as blood, sputum, bronchial cells, airway biopsies and EBC. Exhaled breath condensate has been developed as an alternative to induce sputum (Sastre, Cañas, Rodrigo-Muñoz et al., 2017). However, detection and quantification of miRNAs in this body fluid has been poorly explored. Pinkerton et al. found that miR-21, miR-133, miR-155 and miR-328 in EBC were significantly lower in asthmatic adults (Pinkerton, Chinchilli, Banta et al., 2013). Contrarily, we did not find differences between miRNAs levels among children with or without asthma. Also, miR-21 and miR-155 were not identified as major miRNAs patterns and miR-133a-3p emerged as a biomarker of airway reversibility being positively and significantly associated with a positive bronchodilation without symptoms. Differences in findings may be due to studies heterogeneity in particular related with participants age and different asthma definitions.

Many miRNAs have anti-inflammatory functions and some of them have been shown to be deregulated in asthmatic children (Pinkerton, Chinchilli, Banta et al., 2013). Our study provides further support for miRNAs identifying asthma traits, allowing for a more precise approach on targeted therapy. Particularly, in our study, miR-126-3p identified airway reversibility or self-reported medical diagnosis with reported symptoms in the past 12 months, while it was associated with lower levels of exhaled NO, reflecting an asthma tendentially non-eosinophilic. Also, higher levels of miR-146a-3p were associated with higher response to salbutamol of the small airways making miR-146a-3p a plausible biomarker for the airway reversibility trait when endotyping asthma. Finally, Liu *et al.* and Qin *et al.* reported miR-221 to be upregulated in lymphocytes and serum of asthmatic children, respectively (F. Liu, Qin, Xu et al., 2012; Qin, Xu, Mei et al., 2012), and we have also shown an association with breathing difficulties. Altogether, our results suggested that specific or combined miRNAs may identify asthma and be associated with features and traits of asthma in schoolchildren.

The lungs have been demonstrated to have a very distinctive miRNA profile (Heffler, Allegra, Pioggia et al., 2017) but they have the potential to target hundreds of mRNAs, creating a challenge in identifying their functional roles. Furthermore, abnormal expression of miRNAs may contribute to the development and progression of asthma. Several mechanisms may be associated with this deregulation. For instance, miRNAs were predicted to regulate Th2 cytokines receptors, suggesting that deregulation of this pathway could have implications on the expression and signaling of Th2 cytokines (Pinkerton, Chinchilli, Banta et al., 2013). As such, some miRNAs may be induced in immune cells that were stimulated by pro-inflammatory cytokines. Additionally, others miRNAs may be associated to a negative mechanism of feedback that limit the inflammatory responses by inhibiting NF-kβ (Dubois, Deruytter, Adams et al., 2010; Nejad, Stunden, & Gantier, 2018), which suppresses the expression of mature miRNAs and reduce the inflammatory processes in the airways (Svitich, Sobolev, Gankovskaya et al., 2018) (**Figure 10**).

To evaluate the accuracy in determining the diagnosis for positive bronchodilation with symptoms we performed ROC analysis for the miRNAs combined (data not shown). The participants were divided into one of two groups: acquisition group (n=93) or validation group (n=93). Further, in the acquisition group, we performed a PCA and miRNAs that exhibit factor loadings above 0.5 were considered as significantly contributing to a cluster. Two factors satisfactorily described the distributions of microRNAs levels in children. Additionally, we combined the miRNAs of each PC by adding each microRNA from each PC obtaining 2 scores: the first score was obtained from miR-126-3p, miR-133a-3p, miR-145-5p, miR-221-3p and miR-328-3p; the second included miR-146-5p and miR-423-3p. The AUC value found for the first score, using a RQ value of 0.98, was 0.79 (95%CI= 0.61; 0.97) with a sensitivity of 98.0 % and a specificity of 14.0 % for children without asthma. The last step, included the calculation of the ROC curve for the validation group in which, using the RQ value of the acquisition group, the AUC value was 0.64 (95%CI= 0.44; 0.84) with a sensitivity of 95.6% and specificity of 8.00 %.

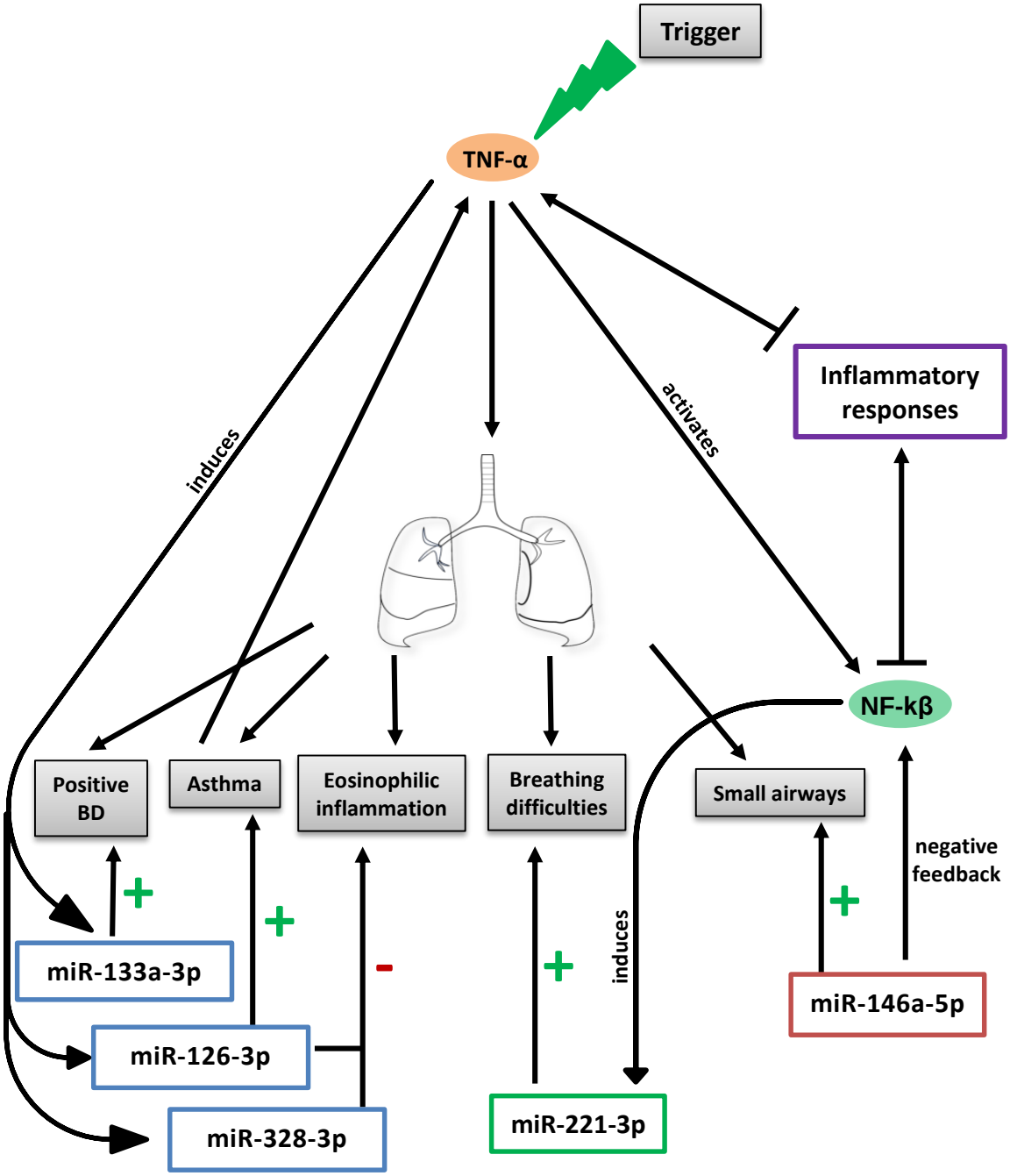


Figure 10. Possible miRNAs network in school aged children. Positive green sign represents a positive association. Negative red sign represents a negative association.

miR: microRNA; NF-kβ: factor nuclear kappa B; TNF-α: tumor necrosis factor alfa; BD: bronchodilation.

6. Conclusion

In conclusion, we showed that the measurement of miRNAs in EBC is feasible, simple, safe and non-invasive. Furthermore, we revealed that individual and specific miRNAs, which were previous described as being deregulated in children with asthma, as well as miRNA profiles, showed potential to identify asthma. We also found associations between miRNAs and specific traits of asthma such as lung function and airway reversibility, eosinophilic airway inflammation and symptoms. As such, these results providing further support for the possibility of using EBC microRNAs as biomarkers of asthma and associated lung function impairment that may assist asthma endotype establishment on the way to a more personalized treatment.

7. Future perspectives

- To study others possible biomolecular mechanisms that may regulate these miRNAs in asthma

- External validity for generalization of our observations, being valid in different age groups.

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