Non-invasive biomarkers in paediatric asthma

SUMMARY

In **chapter 1**, a general introduction on the topic of paediatric asthma and preschool wheeze is given.
Globally, asthma is the most common chronic respiratory disease in adults and children, affecting more than 350 million people. In the past decades, asthma prevalence increased to more than 13% in both children and adolescents, which makes asthma the most common chronic disease in children globally.
Asthma is characterised by respiratory symptoms, such as wheeze and dyspnoea, in combination with a variable and reversible airway obstruction, airway inflammation, and bronchial hyperresponsiveness. However, asthma is a heterogeneous disease, and therefore an adequate and timely diagnosis could be challenging, particularly in young children. In order to prevent misdiagnoses, lung function tests are given a prominent role in the most recent European Respiratory Society (ERS) clinical practice guidelines for the diagnosis of asthma in children aged 5 to 16 years. As these diagnostic tests are not feasible at preschool age, a reliable asthma diagnosis is very difficult in preschoolers. However, asthma symptoms (such as wheeze) are highly prevalent in this age group. As a result, the identification of wheezing preschool children that will remain symptomatic, and thus truly develop asthma, as well as the identification of wheezing preschool children that might benefit from asthma treatment, is a true clinical conundrum.
Therefore, this thesis had 3 main goals. The first objective was to assess the role of non-invasive biomarkers in the prediction of asthma in wheezing preschool children. The second aim was to assess the diagnostic value of exhaled breath analysis for diagnosing asthma in children above 6 years of age. The third goal was to assess the usefulness of exhaled breath analysis in the prediction of a clinical inhaled corticosteroid treatment response in wheezing preschool children.

In **chapter 2**, a comprehensive review was presented on potential (non-)invasive biomarkers which are currently available to predict asthma in wheezing preschool children. A diagnosis of asthma in young children is difficult and cannot be reliably assessed with conventional clinical tools. The study of potential predictive biomarkers in various media, ranging from invasive sampling (e.g. bronchoscopy) to non-invasive sampling (lung function testing and exhaled breath analysis) was comprehensively reviewed. The evolution in biomarker discovery has resulted in an 'omics' approach, in which hundreds of biomarkers in the field of genomics, proteomics, metabolomics and 'breath-omics' can be simultaneously studied.
Many clinical indices, such as the Asthma Predictive Index (API), have been developed, which can be easily used in clinical practice due to the simple measurements. However, their low predictive values demonstrate that additional biomarkers are needed.

Childhood asthma seems to be preceded by diminished lung function in infancy. However, it is yet unclear whether an early diminished lung function is discriminative between transient wheezing children and persistent wheezers prone to develop asthma.
Available studies demonstrated that early sensitisation, severe sensitisation, and persistent sensitisation to common aeroallergens are independent risk factors for developing childhood asthma.
First results on gene expression and exhaled breath profiles in predicting an early asthma diagnosis are promising. This particularly holds for profiles of volatile organic compounds (VOCs) in exhaled breath but could not be established for inflammatory makers in exhaled breath condensate (EBC).
Many hurdles need to be overcome before clinical implementation of new biomarkers is possible. In order to reliably predict asthma in a wheezing child, probably a holistic approach is needed, combining clinical information with blood sampling, lung function tests, and potentially exhaled breath analysis. The further development of predictive, non-invasive biomarkers may eventually improve an early asthma diagnosis in wheezing preschool children, and assist clinicians in early treatment decision-making.

Chapter 3 focussed on biomarkers in exhaled breath condensate as a non-invasive tool to predict and diagnose asthma in children. In this chapter the most recent literature was reviewed and put in perspective.
More than two decades ago, EBC was introduced as a novel, non-invasive tool to assess airway inflammation. This review summarised the latest literature on the various markers in EBC to predict asthma in children. Despite many recommendations and two comprehensive Task Force reports, there is still a large heterogeneity in published data. The biggest issue remains a lack of standardisation regarding EBC collection, preservation, processing, and analysis. As a result, published studies show mixed or conflicting results, questioning the reproducibility of findings. A joint, multi-centre research study is urgently needed to address the necessary methodological standardisation on the above mentioned topics.

Associations between gut microbiota in wheezing children at preschool age and paediatric asthma at age 6 years are given in **chapter 4**.
Reliable biomarkers to predict asthma in wheezing preschool children are lacking. We examined whether gut microbial composition in preschool wheezers was associated with asthma, and investigated associations between gut microbiota and atopic sensitisation, regulatory T-cells (Tregs), and *Foxp*3 gene expression.
In the Asthma DEtection and Monitoring (ADEM) study, 202 wheezing children aged 2-4 years and 50 healthy controls were followed until the age of 6 years, when a diagnosis of asthma was made based on respiratory symptoms and various lung function assessments. At age 2-4 years, faecal microbial composition was analysed by sequencing of

the 16S rRNA V3-V4 gene region. In blood, atopic sensitisation (Phadiatop Infant test), proportion of Tregs ($CD4^+CD25^{high}CD127^-$), and *Foxp*3 gene expression were assessed.
We found no difference in gut microbial diversity, richness, or overall microbial community structure between transient wheezers and true asthmatics, or between preschool wheezers and healthy controls. However, the relative abundance of the genera *Collinsella* (p=0.01) and *Dorea* (p=0.02) was significantly lower in wheezing compared to healthy children, whereas *Gemmiger* (p=0.03) and *Escherichia* (p=0.02) were significantly higher in preschool wheezers who developed asthma. In particular, a high relative abundance of *Escherichia* was associated with a 4.6-fold increased odds of asthma (p=0.02). No significant correlations between abundance of specific bacterial genera and atopic sensitisation, Tregs, and *Foxp*3 gene expression were detected.
In summary, gut microbial diversity and overall gut microbial community structure at preschool age were not associated with wheezing or future asthma. However, some bacterial genera were associated with wheezing or subsequent asthma, suggesting some microbial dysbiosis in children prone for developing asthma.

In **chapter 5**, we presented the results on the feasibility and diagnostic accuracy of a newly developed eNose in children with asthma and cystic fibrosis.
The measurement of VOCs in exhaled breath is a promising tool for diagnosing and monitoring of various lung diseases in children. Gas chromatography mass spectrometry (GC-MS) analysis is a frequently used gold standard technique for VOCs analysis. However, as GC-MS is expensive and time-consuming, hand-held devices or electronic noses (eNoses) have been developed. Recently, the Aeonose was introduced as an easy-to-use handheld eNose capable of point-of-care testing. Although first results using this eNose in adults are promising, studies in children are rare. Therefore, a cross-sectional study in 55 children and adolescents ≥ 6 years of age (20 children with moderate to severe asthma, 13 children with CF, and 22 healthy controls) was performed. The feasibility of the Aeonose was high (> 98% successful measurements). The diagnostic accuracy was high for discriminating asthma from CF (Area Under the Receiver Operating Characteristic Curve [AUC] 0.90 [95% Confidence Interval (CI) 0.78-1.00] sensitivity 89% [65-98%], specificity 77% [46-94%]), and for the distinction between CF and healthy controls (AUC 0.87 [0.74-1.00], sensitivity 85% [54-97%], specificity 77% [54-91%]). However, the diagnostic accuracy for the discrimination between asthma and healthy controls was modest (AUC 0.79 [0.63-0.94], sensitivity 74% [49-90%], specificity 91% [69-98%]). This is the first study to report test results of the Aeonose eNose in children and adolescents ≥ 6 years. This eNose showed a high feasibility with modest to good diagnostic accuracies in asthma and CF.

The influence of inhaled corticosteroids on exhaled VOCs, and the usefulness of exhaled VOCs to predict the clinical response to inhaled corticosteroids (ICS) treatment in preschool children, was discussed in **chapter 6**.
In this study, we investigated the influence of ICS on exhaled VOCs of wheezing preschool children. Furthermore, we assessed whether exhaled VOCs could predict a clinical steroid response in wheezing preschool children. We performed an 8-week ICS crossover trial, in which 147 children were included. Complete data were available of 89 children, of which 46 children were defined steroid responsive based on changes in lung function (airway resistance) and respiratory symptoms. Exhaled VOCs were measured by GC-*tof*-MS. Statistical analysis by means of Random Forest was used to investigate the effect of ICS on exhaled VOCs. A set of 20 VOCs could best discriminate between measurements before and after ICS treatment, with a sensitivity of 73%, and specificity of 67% (area under ROC curve = 0.72). Most discriminative VOCs were branched C11H24, butanal, octanal, acetic acid, and methylated pentane. Other VOCs predominantly included alkanes. Regularised multivariate analysis of variance (rMANOVA) was used to determine treatment response, which showed a significant effect between responders and non-responders ($p<0.01$). These results show that ICS significantly altered the exhaled breath profiles of wheezing preschool children, irrespective of clinical treatment response. Furthermore, exhaled VOCs were capable of determining corticosteroid responsiveness in wheezing preschool children.

Finally, in **chapter 7** a general discussion of the main findings of this thesis, recommendations for future studies, and a discussion on implementation and societal impact was provided.
An estimated half of all preschool children experience at least one episode of wheezing before their sixth birthday. Although in the majority of children, wheezing episodes are transient, one third will have persistent wheeze and possibly develop asthma. Unfortunately, an accurate diagnosis of asthma is not possible in this age group.
This thesis focussed on non-invasive biomarkers, including exhaled breath analysis and gut microbial analysis to predict asthma in wheezing preschoolers. Exhaled breath analysis, e.g. biomarkers of inflammation or oxidative stress in EBC and exhaled VOCs, was introduced decades ago as a novel technique to measure exhaled biomarkers to diagnose and monitor various diseases. The gut microbiome is another highly interesting and non-invasive option, as recent evidence supports the idea of a so-called gut-lung axis. Changes in the gut microbial composition are linked with altered immune responses and homeostasis in the airways.
Our review of currently available literature suggested that biomarkers in EBC are not useful in the prediction of asthma in wheezing preschool children. Moreover, our review once again confirmed the methodological problems of this biological matrix.

In contrast, the analysis of exhaled VOCs seems a more promising tool in predicting asthma in preschool children. In the ADEM study, a set of 17 VOCs was able to predict asthma at preschool age with a high accuracy of 80% after external validation. Most of these VOCs were related to oxidative stress, most probably caused by airway inflammation. When these findings were combined with clinical information (i.e. API) and gene expression, this resulted in a diagnostic accuracy of 95% (positive predictive value 90%, negative predictive value 89%). Although these results are reassuring, these findings need further confirmation and replication. For this purpose, the ADEM2 trial was recently started (Netherlands Trial Register, NL7336). In this trial, a new cohort of wheezing preschool children will be followed to the age of 6 in order to assess the diagnostic potential of exhaled VOCs, by means of various VOCs sensing techniques. Moreover, a randomised controlled trial will be performed to investigate the effect on health gain and reduction in health care costs by using exhaled VOCs for an early asthma diagnosis. Hopefully, the ADEM2 trial will be the next step towards implementing exhaled breath analysis in daily clinical practice in children.

Data from birth cohort studies showed that gut microbial dysbiosis in the first year of life is associated with asthma development. The role of the gut microbiome in the development of asthma after the first year of life was insufficiently explored. To the best of our knowledge, we were the first to explore gut microbial perturbations and its association with asthma at an age (2-4 years) in which the gut microbiome is more mature. Although we found that gut microbial diversity and overall gut microbial community structure at age 2-4 years were not associated with preschool wheezing or future asthma development, a high relative abundance of *Escherichia* in wheezing preschool children was associated with an almost 5-fold increased odds of asthma at age 6. This observation of gut microbial dysbiosis at an older age (*Escherichia* abundance) and the association with future asthma warrants further exploration. The gut microbiome might be an interesting biological matrix when determining asthma risk in preschool children. Infancy is probably the most interesting time frame in which gut microbial perturbations might impact future asthma development. The key to influence the gut microbiome lies in adaptation of our diet, including the (early) supplementation of fibres, and potentially prebiotics and/or probiotics.

Our study on the feasibility and diagnostic accuracy of the Aeonose eNose showed too little diagnostic accuracy in childhood asthma to support broad clinical implementation. The feasibility of the Aeonose in this age group was good. As multiple eNose sensor systems exist, of which the diagnostic performance in asthma or preschool wheezing is currently insufficiently known, future studies should focus on the development and validation of asthma-specific eNoses in children.

In this thesis we found that exhaled breath profiles (VOCs) were significantly different between children who responded to ICS and children who did not respond. This effect was dependent on the future diagnosis of true asthma or transient wheeze at age

6 years, suggesting that wheezing children who eventually developed asthma had a higher chance of being ICS responsive. The treatment of wheezing preschool children, in particular the decision whether or not to start with ICS, has been part of debate for years. For this purpose other biomarkers, such as exhaled nitric oxide (FeNO), blood eosinophilia, and atopic sensitisation, have been studied with varying results. As both preschool wheezing and (paediatric) asthma are too heterogeneous, probably no single biomarker is capable of adequately predicting a clinical ICS response. Our findings showed a novel and innovative potential application of exhaled breath analysis in guiding treatment decisions in children with respiratory symptoms. Moreover, our results could ultimately help in personalising treatment in wheezing preschool children in order to improve cost-effectiveness and reduce side effects.

With respect to a future perspective on exhaled breath analysis, more specifically exhaled VOCs, we recommend to perform basic scientific research focussing on specific confounding factors and the impact of various sampling techniques. However, given the amount of work that need to be done, various research groups active in this field should collaborate and create task forces to address the current knowledge gaps. In a second stage, new (inter)national cohorts in preschool wheeze and paediatric asthma should be formed using GC-MS and other VOCs sensing techniques, in order to perform biomarker discovery with appropriate internal and external validation.

It is unrealistic to believe that the diagnosis of a complex and heterogeneous disease like asthma could be based on one single test or biomarker. The advent of newly developed biomarkers or techniques, such as exhaled VOCs, could aid by improving diagnostic algorithms, better monitoring of disease, and by guiding treatment decisions. This might ultimately lead to an earlier diagnosis of asthma in wheezing preschool children and a reduction in the burden of asthma, with an increased quality of life for our patients.