Research Plan

Phenotyping and outcome predictions by real-time breath analysis with secondary electrospray ionisation-mass spectrometry in children with chronic respiratory symptoms

SHORT TITLE: SESI-MS in children with respiratory symptoms

Type of Research Project:	Research project in which biological material is sampled from humans and/or health-related personal data is collected
Risk Categorisation:	Risk category B
Project Identifier:	SESI MS_CCRS
Project Leader:	PD Dr Alexander Möller Head Respiratory Medicine University Children's Hospital Zürich 8032 Zürich Phone: 0041 44 266 70 79 Fax: +41 44 266 7670 Email: alexander.moeller@kispi.uzh.ch
Health condition / problem	Chronic respiratory symptoms
Project Duration	3/2018 – 8/2024
Project Plan Version and Date:	Version 1.2 (18.06.2018)

ACCESS TO RESEARCH DOCUMENTS

SIGNATURE PAGE(S)

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Project number	SESI MS_CCRS
Project Title	Phenotyping and outcome predictions by real-time breath analysis with secondary electrospray ionisation-mass spectrometry in children with chronic respiratory symptoms

The project leader and the methodologist (PD Dr. med. A. Möller) has approved the research plan version 1 (dated 31.01.2018), and confirm hereby to conduct the project according to the plan, the current version of the World Medical Association Declaration of Helsinki sand the local legally applicable requirements.

Project Leader:

Zürich, den 18.06.2018	PD Dr. med. Alexander Möller
Place/Date	Signature

Project Co-Leader:

Zürich, den 18.06.2018	Prof. Renato Zenobi
Place/Date	Signature

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SYNOPSIS (SUMMARY)

Sponsor/Sponsor-Investigator	PD Dr Alexander Möller
Study Title:	Phenotyping and outcome predictions by real-time breath analysis with secondary electrospray ionisation-mass spectrometry in children with chronic respiratory symptoms
Short Title/Study ID:	SESI-MS in children with chronic respiratory symptoms: SESI-MS_CCRS
Protocol Version and Date:	Version 1 (dated 31.01.2018)
Study Category with Rationale	Risk category B - Research project in which biological material is sampled and/or health-related personal data is collected - Coded data - Non-clinical observational study
Background and Rationale:	Visits for respiratory disease account for 10% of pediatric emergency department visits and 20% of all paediatric hospital admissions. Major respiratory symptoms of respiratory diseases in childhood are wheezing and cough. Both are characteristics of acute and chronic airway diseases, which develop frequently in childhood. Wheezing is the clinical hallmark of obstructive lower airway diseases like asthma, “wheezy” bronchitis or bronchiolitis. Cough is a major characteristic of virtual all lower respiratory infections and may also be the sole presenting symptom of asthma. Whereas most of the children overcome acute episodes of respiratory symptoms without further sequelae, some are at increased risk to develop chronic airway disease. In particular recurrent wheezing in preschoolers has been clearly shown to be a predictor of asthma in school-age and prediction of asthma can be improved when taking chronic cough into account. However, prolonged bacterial airway infection (protracted bacterial bronchitis, PBB) is also characterized by chronic cough and is frequently misdiagnosed as asthma in particular in preschool age. This is manly to the limited objective measurements available in this age range and therefore diagnosis of chronic respiratory disease in preschool children is still challenging. Preliminary results from the Zurich-based collaboration between the University Hospital of Zurich and the Eidgenoessische Technische Hochschule Zürich (ETHZ) have demonstrated the validity and reliability of SESI-MS in evaluating individuals’ exhaled breathprints. Feasibility studies are on-going and clearly show that the technique is applicable in infants and preschool children. There is ample evidence that exhaled breath composition differs between healthy controls and children with respiratory disease and may be useful for non-invasive biomarker analysis.
Objective(s):	The main objective is to provide evidence for the effectiveness of breath anlaysis by SESI-MS in children with respiratory symptoms and to translate the non-invasive, risk-free technique of breath analysis into routine clinical practice.
Outcome: **Primary Outcome** **Secondary Outcome**	*Primary:* Molecular composition of exhaled breath analysed by mass spectrometry *Secondary: Other measurements:* clinical record data, including lung function, allergic sensitization, venous blood markers, throat swab, radiological exams
Study Design:	WP I and WP III: case control studies

	WP II: prospective cohort study
Inclusion/Exclusion Criteria:	Inclusion: *Patients:* more than two episodes of wheeze during 12 months prior to inclusion, daily cough for at least 4 weeks prior to inclusion or doctor diagnosed asthma, age 6 mo. -18 years *Healthy controls:* age 6 mo.-18 years Exclusion *Patients and healthy controls:* heart failure diagnosed after birth affecting pulmonary circulation, specific respiratory diseases: primary ciliary dyskinesia; interstitial lung disease; known significant airway malformation, any disability that prevents proper execution of any measurement manoeuvre (breath analysis, spirometry), fever of at least 38.5°C during the last two weeks prior to the planned first visit No parental consent
Measurements and Procedures:	- Breath analysis by SESI-MS - Clinical records - Questionnaires - Lung Function assessment - Exhaled breath condensate - Exhaled NO - Skin-prick test - Bacteriology (throat swab)
Number of Participants with Rationale (if no Power Analysis conducted):	Total number of children with chronic respiratory symptoms and healthy controls 270 *WP I:* 60 patients with chronic respiratory symptoms with high risk for asthma, 60 patients with chronic respiratory symptoms with low risk for asthma and 60 healthy controls, age range 6mt to 4 years. *WP II:* We aim for a participation of rate 75% (n=135) of the WP I population *WP III:* 90 sex- and age-matched school-aged children: 1. healthy controls (n=30), 2. allergic asthmatics (n=30) and 3. non-allergic asthmatics (n=30). *Rationale:* Traditional methods for estimating sample sizes needed to detect a minimum meaningful difference cannot be applied to our exploratory pilot design. Tentative estimations are made based on sample size requirements for factor analysis and calculations for proteomic profiling using MS.
Study Duration:	03/18-08/2022
Study Schedule:	WP I and WP III: 03/18 – 08/2020 WP II: 03/18-08/2022
Investigator(s):	**Institution:** Universitäts-Kinderspital- Eleonorenstiftung, Fachbereich Pneumologie **Name:** PD Dr. med. Alexander Möller **Address:** Kinderspital Zürich Steinwiesstrasse 75, 8032 Zürich **Email:** alexander.moeller@kispi.uzh.ch **Phone:** 044 266 7079 **Fax:** 044 266 7670
Study Centre(s):	Single-centre: Division of Respiratory medicine University Children's Hospital, Zurich
Statistical Analysis incl. Power Analysis	The SESI-MS data will be acquired and mass calibrated with the Analyst TF 1.7 and PeakView 2.1 software (Applied Biosystems Sciex, Toronto, ON, Canada).

	Demographic and clinical characteristics will be compared using independent-sample pairwise analysis methods (i.e. ANOVA, t-tests, Mann-Whitney U-tests) with Bonferroni-correction for multiple comparisons. Data analysis will be done with Matlab (R2016b and newer, The MathWorks Inc., Natick, MA, US) and R (Development Core Team, R Foundation for Statistical Computing, Vienna, Austria) based on data analysis tools developed at ETH Zurich. As the study approach is exploratory, the possibilities for sample size estimation are limited. Traditional methods for estimating sample sizes needed to detect a minimum meaningful difference cannot be applied to our exploratory pilot design. Tentative estimations are done based on sample size requirements for factor analysis assuming a variable-to-factor ratio of at least seven. We have based our sample size estimation on previous research using SESI-MS.
GCP Statement:	This study will be conducted in compliance with the protocol, the current version of the Declaration of Helsinki, the ICH-GCP or ISO EN 14155 (as far as applicable) as well as all national legal and regulatory requirements.

ABBREVIATIONS

Provide a list of abbreviations used in the project plan - to be completed

BAL	Bronchoalveolar lavage
BMI	Body mass index
CF	Cystic fibrosis
DoH	Declaration of Helsinki
EC	Ethics Committee
EGEP	Essentials of Good Epidemiological Practice
ETHZ	Eidgenössische technische Hochschule Zürich
FEV1	Forced expiratory volume in first second
FVC	Forced vital capacity
FOPH	Federal Office for Public Health
GC-MS	Gas chromatography coupled with Mass Spectrometry
HRA	Federal Act on Research involving Human Beings (Human Research Act, HRA)
HRO	Ordinance on Human Research with the Exception of Clinical Trials (Human Research Ordinance, HRO)
IC	Informed written consent
ID	Identification
IIT	Investigator-initiated Trial
MEF75	Maximum expiratory flow at 75%, 50% and at 25% FVC, resp.
MEF50	
MEF25	
PEF	Peak expiratory flow
PBB	Protracted bacterial bronchitis
SE	Serious event
SESI-MS	Secondary electrospray ionization-mass spectrometry
STROBE	Strengthening the reporting of observational studies in epidemiology
WP	Work packages

SCHEDULE OF ASSESSMENTS (FLOW OF RESEARCH PROJECT)

Project Periods	Screening	Visits						Visits	
Visit	1	2	3	4	5	6	7	1	2 -3
Working package	WP I & II	WP II						WP III	WP IV
Time (age of the patient during the corresponding visit)	Any age between 6 mo.- 4 y.	1y	2y	3y	4y	5y	6y	Any age between 6-18 years	
		The visits can start from any age between 1-6y depending on inclusion age.							
Participant Information and Informed Consent	x							x	
Demographics	x							x	
Medical History	x	x	x	x	x	x	x	x	
In- /Exclusion Criteria	x	x						x	x
Asthma control and respiratory symptoms: Asthma Predictive Index (API)	x							x	x
Questionnaires									
Luftibus (HC)	x							x	
Asthma Control Questionnaire	x	x	x	x	x	x	x	x	
SESI-MS	x	x	x	x	x	x	x	x	x
Spirometry	x			x	x	x	x	x	x
FeNO	x						x	x	x
SPT	x		x	x	x	x	x	x	
Throat swab/sputum	x	x	x	x	x	x	x	x	x

HC: Healthy Control; CRS: child with respiratory symptoms; **PAQLQ:** Paediatric Asthma Quality of Life Questionnaire; **CC-QoL**: Chronic Cough Quality of Life; **PC-QoL**: Chronic Cough Quality of Life,
WP IV Patients from WP III will be asked to return for two additional visits in the case of an asthma worsening. Visit Ex1 will be done within 1 week of start of asthma worsening and Visit Ex2 one month after asthma worsening is resolved.

1. ADMINISTRATIVE STRUCTURE

Sponsor, Project Leader and Coordinating researcher (if identical) to be adapted	Name: *PD Dr. med. Alexander Möller* Address: *Kinderspital Zürich Steinwiesstrasse 75, 8032 Zürich* Email: *alexander.moeller@kispi.uzh.ch* Phone: *044 266 7079* Fax: *044 266 7670*
Project site(s) and responsible researcher:	Institution: *Universitäts-Kinderspital-Eleonorenstiftung, Fachbereich Pneumologie* Name: *PD Dr. med. Alexander Möller* Address: *Kinderspital Zürich Steinwiesstrasse 75, 8032 Zürich* Email: *alexander.moeller@kispi.uzh.ch* Phone: *044 266 7079* Fax: *044 266 7670*
Key Persons involved in research project:	Name: *Tobias Bruderer, PhD* Address: *Department of Chemistry and Applied Biosciences,* ETH Zürich, HCI D 317 CH-8093 Zürich Email: *tobias.bruderer@org.chem.ethz.ch* Phone: *044 632 61 12* Fax: *+41 446 32 12 92* Name: *Prof. Renato Zenobi* Address: *Department of Chemistry and Applied Biosciences,* ETH Zürich, HCI D 317 CH-8093 Zürich Email: *zenobi@org.chem.ethz.ch* Phone: *044 632 43 76* Fax: *+41 446 32 12 92* Name: *Dr. med Christian Bieli* Address: *Kinderspital Zürich Steinwiesstrasse 75, 8032 Zürich* Email: *Christian.bieli@kispi.uzh.ch* Phone: Fax:
Other personnel	See staff list

2. ETHICAL AND REGULATORY ASPECTS

2.1 Ethical Conduct of Study

(HRA Art. 45-49; HRO, Art. 14, 17-23, Annex 2) [1–3]

The research project will be carried out in accordance to the research plan and with principles enunciated in the current version of the Declaration of Helsinki (DoH), the Essentials of Good Epidemiological Practice issued by Public Health Schweiz (EGEP), the Swiss Law and Swiss regulatory authority's requirements as applicable. The EC will be informed about project start and termination.
The research project will only begin once approval from all required authorities has been received.
Any additional requirements imposed by the authorities will be implemented

2.2 Risk categorisation

(HRO Art. 7, 33) [4]

This project is categorized as a non-clinical research project, risk category B. Only procedures with minimal risk and burden (questionnaire, breath collection, lung function, throat and nasal swaps, sputum collection and skin prick testing) are applied. Blood samples will be obtained from venous puncture if clinically indicated.

2.3 Ethics Committee (EC) and Competent Authorities (CA), FOPH

(HRO Art. 14-23, 34, 37, 41, 45) [4]

Before the project will be conducted, the project plan, the proposed participant information and consent form as well as other project-specific documents will be submitted to a competent Ethics Committee (EC).
The regular end, premature end or interruption of the research project is reported to the EC within 90 days upon completion of the project.
Once a year, a list of events or adverse reactions will be submitted to the responsible ethics committee a list of events or adverse reactions and, on this basis, a report on their severity and causal relationship to the intervention, and on the safety of participants (annual safety report).

2.4 Participant Information and Informed Consent

(HRO Art. 8, Annex 2/1.3-1.5) [4]

Participants will be informed about the research project (see below) and consent is sought from each participant. Informed consent will be sought for general and further use of data and/or biological samples.

A) Children with respiratory symptoms:
Parents and patients will be directly informed by the treating physician during clinical visits. Recruitment will be done by the PI, the Co-PI or the research assistants (see staff list). After thorough oral information, informed written consent (IC) about the research project will be obtained from all parents or legal guardians and children older than 14 years who have volunteered to take part in the study. IC will be obtained without delay during the study visit before any study related actions are taken.

B) Healthy controls:
Patients will be recruited from the public and day nurseries. Information by flyers and letters to schools and day care center's (Document Begleitbrief_Schule_gesunde Kontrollen_v1_12.02.2017. Interested parents will contact the study team via the telephone number provided, where they will receive detailed information. At the study date after thorough oral information, informed written consent (IC) about the research project will be obtained from all parents or legal guardians and children older than 14 years who have volunteered to take part in the study. IC will be obtained without delay during the study visit before any study related actions are taken.

Participants will not receive any financial compensation. Travel expenses will be reimbursed on request. As small present will be given to participating children (participation diploma, a small toy and/or a little bag with sweets)

Rationale to include particularly vulnerable participants:

We aim to identify and validate the most specific metabolites in exhaled air to reliably and non-invasively diagnose both early infection and inflammation processes in the lungs of children with respiratory symptoms. This will help to understand the early pathologic processes of the disease.
Due to limited objective measurements available for preschool children proper diagnosis of chronic respiratory disease is very challenging in this age range. This has important implications on disease morbidity and outcome as treatment and preventive measures depend on the correct diagnosis. To date many children are treated with repeated or long-term inhaled corticosteroids with minimal effect as do not suffer from asthma. On the other hand, children with asthma are not treated sufficiently to prevent long term sequela. Better phenotyping of children with chronic respiratory complaints allows correct and efficient therapy and will lead to personalized treatment approaches reducing side effects, improve efficacy and reduce disease burden and health costs. We aim to apply the methodology of SESI-MS to characterize early and non-invasively airway inflammation and metabolic processes due to airway inflammation.

This is an observational study. Hence, no health risks are expected. All tests and measurements are associated with no or minimal risks for the participants.

2.5 Participant privacy and safety

(HRA Art. 1, Annex 2/1.7) [2]
The Project Leader affirms and upholds the principle of the participants' right to dignity, privacy and health and that the project team will comply with applicable privacy laws. Especially, anonymity of the participants will be guaranteed when presenting the data at scientific meetings or publishing them in scientific journals.
Individual participant medical information obtained as a result of this research project is considered confidential and disclosure to third parties is prohibited. Participant confidentiality will be further ensured by utilising identification code numbers to correspond to medical information in the computer files.
For data verification purposes, authorised representatives of the Sponsor, a competent authority (e.g. FOPH), or an ethics committee may require direct access to parts of the medical records relevant to the project, including participants' medical history..

2.6 Early termination of project

(HRO Art. 22) [4]

The regular end, premature end or interruption of the research project is reported to the EC within 90 days upon completion of the project.
The project will be terminated prematurely in case of insufficient participant recruitment.

2.7 Amendments, Changes

(HRO Art. 18, 22, Annex 2) [4]

Significant changes to the project will be submitted as an amendment according to according HRO Art. 18 to this application to the responsible EC by the Project Leader providing information on the reasons for the change.
Substantial amendments are only implemented after approval of the EC.

3. INTRODUCTION

(STROBE 2; HRO Annex 2/1.2) [4–6]

3.1 Background

Describe the research question, including any scientific data (and if relevant any pre-/clinical evidence) on which the research project is based (published / unpublished) and including disease background, e.g. distribution in population, current standard of care (if relevant).
Any statements that rely on existing knowledge or published information shall be adequately referenced.

3.1.1 The burden of respiratory diseases in children

Visits for respiratory disease account for 10% of pediatric emergency department visits and 20% of all paediatric hospital admissions [7]. The prevalence of asthma, the most common chronic respiratory disease in childhood, increased from 3.6% in 1980 to 5.8% in 2003 among U.S. children and asthma is the third leading cause of hospitalization among persons under 18 years of age in the United States [8]. Similar figures have been show for Swiss children, where the prevalence was 7.5% in 2001 [9]. Major respiratory symptoms of respiratory diseases in childhood are wheezing and cough. Both are characteristics of acute and chronic airway diseases, which develop frequently in childhood. Wheezing is the clinical hallmark of obstructive lower airway diseases like asthma, “wheezy” bronchitis or bronchiolitis. Cough is a major characteristic of virtual all lower respiratory infections and may also be the sole presenting symptom of asthma [10,11].
Whereas most of the children overcome acute episodes of respiratory symptoms without further sequelae, some are at increased risk to develop chronic airway disease. In particular recurrent wheezing in preschoolers has been clearly shown to be a predictor of asthma in school-age and prediction of asthma can be improved when taking chronic cough into account [12–14]. However, prolonged bacterial airway infection (protracted bacterial bronchitis, PBB) is also characterized by chronic cough and is frequently misdiagnosed as asthma in particular in preschool age [15]. This is manly to the limited objective measurements available in this age range and therefore diagnosis of chronic respiratory disease in preschool children is still challenging.

3.1.2 Preschool wheeze and development of asthma later in life

Asthma is the most common chronic airway disease in childhood. Besides being the cause of extensive healthcare costs asthma represents a physical and psychological burden for the affected children and their families. In the course of the last century atopic diseases showed a steady increase in prevalence [16]. The onset of asthma varies by age and depends on genetic background and environmental exposure [17,18]. Early diagnosis is associated with improved symptom control, function in every day life and decrease of risk of exacerbations [19]. Moreover, early structural changes in bronchoscopy studies in severe preschool wheezers and follow-up of long-term cohort studies suggest an association between childhood asthma and early airway remodelling and ultimately development of chronic obstructive pulmonary disease later in life [20].
International guidelines exist for asthma diagnosis, treatment and monitoring [21]. However, spirometry, as the gold standard to assess airway obstruction, depends on well executed forced exhalation manoeuvres that are largely impossible in children younger than four years old. Thus, diagnosis of asthma in this age group still is challenging depending mainly on patient history and assessment of risk factors (e.g. allergy tests). Wheeze, as the main clinical feature of lower airway obstruction, is highly unspecific in the preschool age due to phenotype variability. While wheezing children will grow out in most of the children by school age, it persists in some and as a chronic disease we call asthma [12]. Clinical phenotyping of preschool wheeze remains a crucial task for optimal disease management. While the early transient wheeze phenotype is mainly treated symptomatic, for late onset and persistent wheezing - both associated with high risk for asthma persistence until school age - symptom control is mainly established by preventive therapy with inhaled corticosteroids (ICS) [19].
The lack of objective measures for phenotyping asthma in preschool age stimulated a whole area of research and several methods for assessment airway function and inflammation have bee investigated. Infant lung function can be seen as a surrogate for spirometry in older children. However, the procedures are time-consuming and only applicable in sedated infants thereby limiting its application in clinical routine. Atopy is a well-known risk factor for development and persistence of asthma. Although the degree of allergen specific immunoglobulin E (IgE) to common allergens has been repeatedly shown to be associated with asthma risk, negative predictive values are higher than positive ones, indicating that these models are better at excluding asthma than at predicting it [22]. Eosinophil counts in in sputum or

bronchial lavage samples are and increased in asthma and its quantification represents a potential marker for asthma risk. However, its collection requires patient cooperation (sputum) or invasive methods (bronchoalveolar lavage, BAL) thereby limiting its clinical use. There is a urgent need for non-invasive, sensitive measurements for establishing an asthma diagnosis in the preschool age.

3.1.3 Chronic cough and protracted bacterial bronchitis in children

A child coughing daily more than four weeks is considered to have chronic cough [23]. Chronic cough is associated with significant morbidity and a burden for parents [24,25]. The assessment of chronic cough in childhood is challenging because of (i) the high prevalence and low discrimination between mild and severe disease, (ii) the multitude of potential aetiologies, and (iii) the limited objective measurements available (in particular in preschool children). Whereas asthma may cause chronic cough, it is largely overdiagnosed in these children [26]. The most important cause of chronic cough in childhood is PBB, a chronic bacterial infection of the lower airways. PBB represents the beginning of the disease spectrum from PBB to chronic suppurative lung disease to bronchiectasis, the irreversible and potentially life limiting end stage [27]. Timely diagnosis and treatment is likely to halt disease progression [28]. However, diagnosis of PBB is cumbersome. To date it is widely seen as a diagnosis of exclusion with the most reliable “test” being a successful treatment response with a prolonged course of antibiotics over several weeks. BAL might allow an earlier diagnosis, but is too invasive to be used in clinical practice. Therefore, there is potential to confirm the diagnosis of PBB at an earlier stage of the disease, reassure parents and doctors, and prevent overtreatment.

3.1.4 Exhaled breath in children with respiratory diseases

3.1.4.1 Exhaled breath condensate (EBC)

EBC has been extensively studied as a technique for identifying biomarkers in different pulmonary diseases. Whilst EBC is easily collected by cooling the exhaled air and trapping the resulting condensation which is assumed to represent the diluted composition of airway lining fluid, its value as a clinical tool is currently hampered by a lack of standardisation, by high intra-subject variability and low validity [29–31]. As a sampling method for airway lining fluid, EBC – compared to invasive techniques like BAL, bronchoscopy or biopsy – seems promising, particularly in children. But the impact of methodological and sampling pitfalls must not be underestimated [32,33]. Currently investigated biomarkers in EBC include pH [34], urea [35], nitric oxide metabolites [36], reactive oxygen species [37], matrix metalloproteinases [38], cytokines [39], 8-isoprostane [40], nitrotyrosine [41] and others. An obvious shortcoming of EBC analysis is its failure to capture volatile compounds not soluble in water.

3.1.4.2 Isolated Gaseous Components

Of all exhaled biomarkers, fraction of exhaled nitric oxide (FeNO) is the most studied biomarker and is the only exhaled biomarker with widespread clinical use. FeNO is considered to be a non-invasive marker of eosinophilic airway inflammation [42]. Whilst a standardized, online FeNO sampling technique is used in schoolchildren, unstandardized and offline FeNO sampling is mostly applied in preschool children [43]. We could previously show that FeNO levels were higher in wheezing preschool children with probable asthma compared to preschool children without symptoms or with probable transient wheezing symptoms [44]. However, the study had a cross-sectional design and used ‘probable asthma’ as a diagnosis. In a longitudinal cohort, it was found that a FeNO concentration of ≥ 30 ppb at preschool age could predict persistence of wheezing symptoms at three years (sensitivity 77%, specificity 94%) [45]. Accordingly, we could demonstrate in a prospective cohort study with 391 preschool children (aged three months to four years) that FeNO was elevated in preschool children who developed asthma at school age compared to children without later asthma [46]. In the PIAMA study, it was demonstrated that FeNO and IgE (but not airway resistance) measured at four years of age were predictive for asthma symptoms at age eight, independent of clinical his- tory [47]. In a subsequent analysis of this cohort, FeNO was related to the classic wheezing phenotypes with increased levels in persistent wheezing compared to transient wheezing phenotypes [12,48]. However, elevated FeNO in persistent wheezers was only present in children with allergic sensitization [49]. As presence of atopy seems to modify the relationship between FeNO and wheezing phenotypes, a different pathophysiology of wheeze in atopic and non-atopic children might be present. Potentially, eosinophilic inflammation might play a predominant role in atopic persistent wheezers, whilst other types of inflammation such as neutrophilic inflammation might play an important role in non-atopic persistent wheeze. As FeNO compromises only one (eosinophilic) biomarker, it might not be sufficient to study the broad range of preschool wheezers, and additional biomarkers are needed to be studied.

3.1.4.3 Whole breath analysis

Human tidally exhaled air contains contributions of about 150 ml of dead space and 350 ml of alveolar respiration. Exhaled breath contains more than 3,500 identified and many more unidentified components. Most of exhaled breath volume is constituted by gases like O_2, NO, and CO_2. Additionally, there is a large number of VOCs, present in minute quantities whose analysis by GC-MS was pioneered by Linus Pauling's group in the 1970ies [50,51].

Real-time analysis of so-called breathprints (exhalome) via MS is a highly promising but little explored area of translational research. Rather than comparing only small numbers of pre-selected or highly salient compounds, breathprints represent relative intensity patterns (so-called mass-to-charge or m/z pairs) for a multitude compounds reflecting subject-specific metabolic signatures. Potential future applications include internal body clock estimation, disease risk prediction and monitoring of treatment adherence [52]. Secondary electrospray ionisation-mass spectrometry (SESI-MS) has been developed for that purpose [53]. It can detect differences between individual breathprints that are reasonably stable over time and follow predictable diurnal patterns [54,55]. SESI-MS' simple non-invasive set-up merely requires persons to exhale through a sterile plastic mouthpiece into a heated tube making it highly appealing as a clinical tool. So far, SESI-MS breathprint analysis has not been applied to children with respiratory complaints.

Our study will be one of the first to analyse whole breath molecular composition rather than investigate isolated gases or molecules. In contrast to previous studies, this unbiased approach enables the identification of disease-specific breath patterns including thousands of volatile compounds. Our method allows for the simultaneous analysis of tiny concentrations of molecules, many of which have hitherto not yet been molecularly characterised.

3.1.4.4 Recent finding on breath analysis in children with respiratory diseases

Recently, the use of mass spectrometry in breath analysis in respiratory disease gained more attention due to major technical advances. In a case-control study in 120 Dutch children exhaled breath was analysed by gas chromatography coupled with Mass Spectrometry (GC-MS). Breath analysis could accurately distinguish children with asthma from healthy children, achieving a sensitivity of 89% and a specificity of 95% [56]. Two authors from a Dutch research group recently published prospective data from the ADEM (Asthma DEtection and Monitoring) cohort, where preschool children were followed from three to six years of age and breath profiles were repeatedly measured by GC-MS [57,58]. With a set of volatile organic compounds (VOCs) the population could be classified into healthy, transient wheezing and asthmatic with reasonable accuracy.

Until now no studies in children with PBB and/or chronic cough have been conducted. However, there are a small number of studies in cystic fibrosis (CF), a inherited lung dieseases that is characterized with chronic microbial infection and thereby resembling PBB. Robroeks et al. compared 48 CF and 57 control subjects who exhaled into plastic bags [59]. Breath-borne particles were immobilised before GC-MS analysis. Different breathing manoeuvres did not alter VOC patterns which reliably predicted CF status and Pseudomonas colonisation. Barker et al. followed a similar approach to study selected VOCs in 20 CF patients and 20 healthy controls [60]. They found good intra-subject reproducibility and associations with airflow limitation and Pseudomonas status.

A 2012 review of breath analysis in CF summarises its ability of identifying microbial airway colonisation, differentiation of CF from asthma and healthy controls, identification of steroid-naïve vs. steroid-treated CF patients and recognition of stable vs. acutely exacerbated CF patients [61]. However, all the earlier breath analysis data have to be interpreted in the light of several limitations. As breath was analysed by gas chromatography and real-time (as in SESI-MS), breath was collected and storaged in inert bags. Moreover, this technique requires sample pre-processing is necessary before analysis and issues like sample contamination and molecule degradation due storage and pre-processing are critical issues. SESI-MS not only circumvents these issues by real-time measurement, but also detects molecules with high sensitivity in a larger range than other mass spectrometry techniques [76, 77, 78].

3.2 Rationale for the research project

Preliminary results from the Zurich-based collaboration between the University Hospital of Zurich and the Eidgenoessische Technische Hochschule Zürich (ETHZ) have demonstrated the validity and reliability of SESI-MS in evaluating individuals' exhaled breathprints [52,55]. Feasibility studies are on-going that clearly show that the technique is applicable in infants and preschool children. There is ample evidence that exhaled breath composition differs between healthy controls and children with respiratory disease and may be useful for non-invasive biomarker analysis.

The main objective is to provide evidence for the discriminative capacity of breath analysis by SESI-MS in children with respiratory symptoms and to translate the non-invasive, risk-free technique of breath

analysis into routine clinical practice. Due to limited objective measurements available for preschool children proper diagnosis of chronic respiratory disease is very challenging in this age range. This has important implications on disease morbidity and outcome as treatment and preventive measures depend on the correct diagnosis. To date many children are treated with repeated or long-term inhaled corticosteroids with minimal effect as do not suffer from asthma. On the other hand, children with asthma are not treated sufficiently to prevent long term sequela. Better phenotyping of children with chronic respiratory complaints allows correct and efficient therapy and will lead to personalized treatment approaches reducing side effects, improve efficacy and reduce disease burden and health costs. We aim to apply the methodology of SESI-MS to characterize early and non-invasively airway inflammation and metabolic processes due to airway inflammation.

Our research hypotheses are:

- The development of asthma later in life can be predicted by specific breath prints and/or specific molecules in exhaled breath in preschool children with chronic respiratory symptoms
- The diagnosis of protracted bacterial bronchitis can be preponed by breath analysis in preschoolers with chronic cough
- Breath analysis is capable to classify school-aged children with and without asthma (breath prints and/or specific molecules in exhaled breath)
- Specific breath prints predictive for asthma in school age can also be found in preschool children that are likely to develop asthma later in life
- Controlled and uncontrolled asthma can be distinguished by breath analysis in school-aged children
- Phenotyping of asthma based on specific breathprints is possible in school age children

3.3 Risk-Benefit Assessment

(HRA Art. 12; HRO Art. 15) [2,4]

This is an observational study. Hence, no health risks are expected. All tests and measurements are associated with no or minimal risks for the participants. The benefit of the study is the information gained on the development of chronic respiratory symptoms in childhood. Those children enrolled in the study who develop a respiratory disease will profit from a close monitoring along the study and benefit of an early diagnosis. Healthy participants might help family members or other children in having better knowledge and understanding for respiratory diseases.

To date, it is rarely possible to make a correct asthma diagnosis in preschool children presenting with chronic airway symptoms. In addition, there is no single predictor for asthma in wheezy pre-schoolers. This has important implications on disease morbidity and outcome as treatment and preventive measures depend on the correct diagnosis. To date many children are treated with repeated or long-term inhaled corticosteroids with minimal effect as do not suffer from asthma. On the other hand, children with asthma are not treated sufficiently to prevent long term sequela. Better phenotyping of children with chronic respiratory complaints allows correct and efficient therapy and will lead to personalized treatment approaches reducing side effects, improve efficacy and reduce disease burden and health costs.

All measures to prevent unauthorized data access will be taken. The Project Leader affirms and upholds the principle of the participants' right to dignity, privacy and health and that the project team will comply with applicable privacy laws. Especially, anonymity of the participants will be guaranteed when presenting the data at scientific meetings or publishing them in scientific journals.
Individual participant medical information obtained as a result of this research project is considered confidential and disclosure to third parties is prohibited. Participant confidentiality will be further ensured by utilising identification code numbers to correspond to medical information in the computer files.

4. OBJECTIVES, ENPOINTS/OUTCOMES AND OTHER STUDY VARIABLES

4.1 Objectives

(STROBE 3) [6]

Zürich Exhalomics is a flagship project of the Universities of Zürich (University of Zürich and ETHZ) and the department of paediatric pneumology of the University Children's Hospital Zürich is part of this interdisciplinary consortium. We plan a clinical study to investigate specific breath prints and molecules for the assessment and prediction of children with chronic respiratory symptoms by breath analyses with SESI-MS.

We plan to reach this aim by answering following research questions:

- Do breath prints separate healthy preschool children differ from their peers with respiratory symptoms? (WP I, see below)
- Do breath profiles differ among symptomatic preschool children? (WP I)
- Is there an association between breath profiles and conventional clinical phenotypes? (WP I)
- Is breath analysis by SESI-MS in preschool age diagnostic for PBB? (WP I)
- Do the breath profiles in preschool age predict outcomes later in life (asthma, transient wheeze)? (WP I & II)
- Do breath prints differ between healthy school-aged children differ and their asthmatic peers diagnosed by conventional methods (clinics, lung function, allergy testing)? (WP III)
- Can SESI-MS distinguish between controlled and uncontrolled asthma? (WP III)
- Can phenotyping by SESI-MS in school-age children be replicated in a second population? (WP II & III)
- Which single biomarkers for distinct respiratory diseases can be extracted from breath profiles from preschool and school-aged children? What is their role in disease pathogenesis? (WP I, II & III)
- How can SESI-MS analysis be streamlined for routine clinical use? (WP I, II & III)
- Are breathprints associated with microbial colonisation, symptoms, and clinical history? (WP I, II & III)

Our research hypotheses are:

- The development of asthma later in life can be predicted by specific breath prints and/or specific molecules in exhaled breath in preschool children with chronic respiratory symptoms
- The diagnosis of protracted bacterial bronchitis can be preponed by breath analysis in preschoolers with chronic cough
- Breath analysis is capable to classify school-aged children with and without asthma (breath prints and/or specific molecules in exhaled breath)
- Specific breath prints predictive for asthma in school age can also be found in preschool children that are likely to develop asthma later in life
- Controlled and uncontrolled asthma can be distinguished by breath analysis in school-aged children
- Phenotyping of asthma based on specific breathprints is possible in school age children

4.2 Primary and secondary endpoint/outcome(s)

(STROBE #7) [6]

4.2.1 Primary endpoints

The primary outcome is the molecular composition of tidally exhaled breath as analysed in real-time by SESI-MS.

4.2.2 Secondary endpoints

Secondary endpoints consist of already established methods for subjective and objective quantification of respiratory pathology:

- Asthma control and respiratory symptoms: Asthma Predictive Index (API), Asthma Control Questionnaire [62]

- Lung function (spirometry): Forced vital capacity (FVC), forced expiratory volume in first second (FEV1), peak expiratory flow (PEF), FEV1/FVC ratio, maximum expiratory flow at 75%, 50% and at 25% FVC (MEF75, MEF50, MEF25) and MEF75–25 ratio
- Exhaled NO
- Allergy testing: Skin prick testing and serologic testing
- Questionnaires: Respiratory symptoms, personal and family history regarding airway and lung disease, environmental exposures, socioeconomic status.
- Anthropometrics: Age, weight, length, and body mass index (BMI)
- LuftiBus questionnaires for healthy controls: 1. Comorbidities, personal and family history in that regard, environmental exposures, socioeconomic status. 2. To assess the respiratory health status of the healthy controls (KEK-ZH-Nr. 2014-0491).
- Exhaled breath condensate

4.3 Other study variables

(STROBE #7) [6]

Assessment of potential confounders:

- bacterial colonization of upper airways
- exposure to cigarette smoke

5. PROJECT DESIGN

(STROBE 4,5, 9; HRO Annex 2/1.2) [4,6]

5.1 Type of research and general project design

(STROBE 4)

Type: observational

Single center

Design:

WP 1 / WP lll	Cross sectional case control study
WP ll	Longitudinal cohort study
WP IV	longitudinal assessment of asthma worsening

Our project will consist of four work packages (WP):

WP I 'preschool'; prospective cross-sectional exploratory observational matched case control study:
WP I will consist of age and sex matched case-control study including 180 preschool children aged 6 months to 4 years. It is designed for phenotyping children with chronic respiratory symptoms by SESI-MS.
Three groups will be investigated:
Healthy controls (n=60) and two groups cases stratified by diagnostic certainty: A) patients with chronic respiratory symptoms (wheezing, cough) and low risk for asthma (n=60) and B) patients with chronic respiratory symptoms (wheezing, cough) and high risk for asthma (n=60).
Asthma risk will be assessed applying the modified asthma predictive index (mAPI) and the Leicester prediction tool [14,66].

WP II 'longitudinal'; prospective longitudinal observational cohort study: For prospective assessment of health outcomes parents of the participants of WP I will be asked to take part in longitudinal follow-up study. Participants will be followed until reaching the age where lung function testing reliably can be conducted (six years). Therefore, the duration of the follow depends on the age at study entry (two to six years). Two follow-up schemes will be offered:

1. This scheme consists of one follow-up visit at the age six. We aim for a participation of rate 75% (n=135) of the WP I population.
2. For this scheme parents will be offered yearly follow-up visits until the age of six. We aim to recruit at least 45 healthy controls, 45 patients with chronic respiratory symptoms (wheezing, cough) and low risk for asthma, and 45 patients with chronic respiratory symptoms (wheezing, cough) and high risk for asthma

WP III 'school-age'; prospective cross-sectional exploratory observational matched case control study: In this case-control study we enrol three groups with sex- and age-matched school-aged children (age 5 - 18 years): A) healthy controls (n=30), B) children with known allergic asthma (n=30) and C) children with known non-allergic asthma (n=30).
Patients are recruited from the outpatient clinic of our department at regular asthma follow-up. Control subjects will be recruited from public schools. The inclusion criteria are age 5 to 16 years and German or English speaking. Asthmatics have to be controlled according the modified asthma control questionnaire [67]. Patients are asked to withdraw any asthma control medication three weeks before the study visit (in accordance with international asthma control guidelines) [68].

WP IV: 'school-age' assessment during asthma worsening. Patients from WP III will be asked to return for two additional visits in the case of an asthma worsening. Visit Ex1 will be done within 1 week of start of asthma worsening and Visit Ex2 one month after asthma worsening is resolved. Patients with the need of an acute treatment with systemic corticosteroids or hospitalization will not be included in this part.

5.2 Procedures

(STROBE 5; swissethics 3a) [6,69]

Work package I: 'preschool'

Step 1: recruitment (see 2.4). During the recruitment besides the parent information leaflets and consents, the questionnaire will be distributed to parents. They will be asked to bring the filled-in Luftibus questionnaire on the day of SESI-MS measurement. Asthma risk will be assessed applying the modified asthma predictive index (mAPI) and the Leicester prediction tool to categorise patients to high or low asthma risk groups.

Step 2: measurement visit: All children will undergo a short clinical assessment by the attending physician. Weight and body-length will be measured as per clinical requirements. Information on actual treatment and last medication (if applicable), last food intake will be gathered. Participants will be thoroughly examined for atopic dermatitis during the medical visits, using the SCORing Atopic Dermatitis method (SCORAD), performed by a medical doctor.

All measurements will be performed at the Kinderspital Zurich.

Prior to the measurement, participants will need to have abstained for at least one hour from passive smoking, eating (including chewing gum), beverages (except for plain water), brushing of teeth and using mouthwash. This should cause minimal discomfort, as participants are free to indulge in normal daily activities and dietary habits up to one hour before the beginning of the test session and immediately after its completion.

SESI-MS measurement:

- Children 1.5-4 years of age: for the breath analysis children are comfortably seated on a chair or on the lap of the parent and will be asked to breathe calmly as they usually breathe for about 15 seconds through a sterile mask into the mass spectrometer.
- Infants <1.5 years of age: measurements will be performed while the child is in natural sleep. The child will be in supine position either on the lap of the mother or in the cot. The face mask will be carefully placed over the nose and mouth of the sleeping child. This procedure is entirely risk-free as no alteration of normal breathing is required. After six breath cycles of 15 seconds over a period of several minutes, measurement is complete.

Allergy testing:
Skin-prick test (SPT) for the 7 most common aeroallergens in Switzerland (*D. pteronissimus*, cat, grass mix, birch, hazelnut, ash, and alder) will be performed in all healthy controls and children with respiratory symptoms ≥2y whom allergy testing has never been performed before. Results from allergy testing for most of the patients with respiratory symptoms is available routine clinical laboratory testing.
SPT is a simple, safe and quick test, providing results within 15-20 minutes. A drop of the allergen (extract) solution is placed on the skin of child comfortably seated on a chair or on the lap of the parent. The skin is then pricked through the drop using the tip of a lancet – this can feel a little sharp but is not painful and should not bleed. In case of sensitization to an allergen it elicits a small, localised allergic response, in the form of a wheal (bump) and flare (redness) at the site of testing, which disappears within 15 minutes. In the standard procedure local antihistamine gel is applied after the test to avoid itching.

Throat swab
Airway and oral bacteria are metabolically active and may add to the exhaled volatile metabolites. Therefore, airway cultures need to be assessed in all children. Throat swab: a small cotton will be used to swab the posterior nasopharynx and then placed into the culture medium.

Fractional exhaled Nitric oxide (FeNO):
The fraction of exhaled nitric oxide (FeNO) is a non-invasive biomarker associated with airway inflammation and can be measured across all ages in standardized fashion. For the reservoir-offline technique, children are sitting on the legs of the parent or a chair while tidally breathing NO-free air for ten breaths. A reusable inert collection bag is attached to the expiratory side of a non-rebreathing valve

with a resistor in place. Five breaths during relaxed tidal breathing will be sampled, and FeNO will be analysed within 4 hours using a chemiluminescence analyser. FeNO will be assessed in healthy controls only.

The whole procedure will take 40 minutes.

Work package II 'longitudinal':

Step 1: all the subjects from WP I will be asked to continue their participation in WP II. The scheme of the visits will be presented to parents

Step 2; Interim visits (yearly): all children will undergo a short clinical assessment by the attending physician. Weight and body-length will be measured as per clinical requirements. Information on actual treatment and last medication (if applicable), last food intake will be gathered. Participants will be thoroughly examined for atopic dermatitis during the medical visits, using the SCORing Atopic Dermatitis method (SCORAD), performed by a medical doctor.

All measurements will be performed at the Kinderspital Zurich.

Prior to the measurement, participants will need to have abstained for at least one hour from passive smoking, eating (including chewing gum), beverages (except for plain water), brushing of teeth and using mouthwash. This should cause minimal discomfort, as participants are free to indulge in normal daily activities and dietary habits up to one hour before the beginning of the test session and immediately after its completion.

SESI-MS measurement:

- Children 1.5-4 years of age: for the breath analysis children are comfortably seated on a chair or on the lap of the parent and will be asked to breathe calmly as they usually breathe for about 15 seconds through a sterile mask into the mass spectrometer.
- Infants <1.5 years of age: measurements will be performed while the child is in natural sleep. The child will be in supine position either on the lap of the mother or in the cot. The face mask will be carefully placed over the nose and mouth of the sleeping child. This procedure is entirely risk-free as no alteration of normal breathing is required. After 6 breath cycles of 15 seconds over a period of several minutes, measurement is complete.
- Children ≥4 years of age: measurements will be performed via a mouthpiece. Comfortably seated participants will be asked to exhale calmly for about 15 seconds through a sterile plastic mouth piece into the MS. This procedure is entirely risk-free as no alteration of normal breathing is required – it is less demanding than routine spirometric testing. After six repeated exhalations over a period of several minutes, testing is complete.

Spirometry

Lung function will be measured in all children ≥4 years of age. In the case of a recent lung function measurement (less than 2 weeks prior to the study date) no spirometry will be performed. This is a standard procedure routinely used in medical praxis to assess the lung function. The children are asked to take the deepest breath they can, and then exhale into the lung function mouth piece as hard as possible, for as long as possible, preferably at least 6 seconds. During the test, soft nose clips may be used to prevent air escaping through the nose. Sterile single use filter mouthpieces are used to prevent the spread of microorganisms.

Fractional exhaled Nitric oxide (FeNO):

The fraction of exhaled nitric oxide (FeNO) is a non-invasive biomarker associated with airway inflammation and can be measured across all ages in standardized fashion. For the reservoir-offline technique, children are sitting on the legs of the parent or a chair while tidally breathing NO-free air for ten breaths. A reusable inert collection bag is attached to the expiratory side of a non-rebreathing valve with a resistor in place. Five breaths during relaxed tidal breathing will be sampled, and FeNO will be analysed within 4 hours using a chemiluminescence analyser.

Throat swab

Airway and oral bacteria are metabolically active and may add to the exhaled volatile metabolites. Therefore, airway cultures need to be assessed in all children. Throat swab: a small cotton will be used to swab the posterior nasopharynx and then placed into the culture medium

Exhaled breath condensate:
EBC are collected by a simple glass cold trap which is placed into a cooling sludge (T = -78.5°C, isopropanol/dry ice) following the guidelines by the ATS/ERS task force [77]. The cold trap is fixated on a table for safety and an exhaust line is applied to the cooling sludge to avoid any possible fumes from the isopropanol. The child breathes through a disposable, sterile mouthpiece into a teflon line connected to the cold trap during 5-15 minutes. The samples are immediately stored at -80°C until up-concentration by lyophilisation and analysis with ultra- high performance liquid chromatography high resolution tandem mass spectrometry (UHPLC-HRMS/MS) at either the Kinderspital Zurich or ETH Zurich.

Step 2; the last visit: all the procedures from interim visits will be repeated. In addition the second SPT will be performed to healthy controls and children with respiratory symptoms who don't have any allergy testing within last 3 months.

WP III 'school-age'

Step 1: recruitment (see 2.4). During the recruitment besides the parent information leaflets and consents, the questionnaires will be distributed to parents. They will be asked to bring the filled-in questionnaires on the day of SESI-MS measurement. Asthma risk will be assessed applying the modified asthma predictive index (mAPI) and the Leicester prediction tool to categorise patients to high or low asthma risk groups.

Step 2: measurement visit: All children will undergo a short clinical assessment by the attending physician. Weight and body-length will be measured as per clinical requirements. Information on actual treatment and last medication (if applicable), last food intake will be gathered. Participants will be thoroughly examined for atopic dermatitis during the medical visits, using the SCORing Atopic Dermatitis method (SCORAD), performed by a medical doctor.

All measurements will be performed at the Kinderspital Zurich.

Prior to the measurement, participants will need to have abstained for at least one hour from passive smoking, eating (including chewing gum), beverages (except for plain water), brushing of teeth and using mouthwash. This should cause minimal discomfort, as participants are free to indulge in normal daily activities and dietary habits up to one hour before the beginning of the test session and immediately after its completion.

SESI-MS measurement:
Measurements will be performed via a mouthpiece. Comfortably seated participants will be asked to exhale calmly for about 15 seconds through a sterile plastic mouth piece into the MS. This procedure is entirely risk-free as no alteration of normal breathing is required – it is less demanding than routine spirometric testing. After six repeated exhalations over a period of several minutes, testing is complete.

Spirometry
Lung function will be measured in all children ≥4 years of age. In the case of a recent lung function measurement (less than 2 weeks prior to the study date) no spirometry will be performed. This is a standard procedure routinely used in medical praxis to assess the lung function. The children are asked to take the deepest breath they can, and then exhale into the lung function mouth piece as hard as possible, for as long as possible, preferably at least 6 seconds. During the test, soft nose clips may be used to prevent air escaping through the nose. Sterile single use filter mouthpieces are used to prevent the spread of microorganisms.

Fractional exhaled Nitric oxide (FeNO):
The fraction of exhaled nitric oxide (FeNO) is a non-invasive biomarker associated with airway inflammation and can be measured across all ages in standardized fashion. For the reservoir-offline technique, children are sitting on the legs of the parent or a chair while tidally breathing NO-free air for ten breaths. A reusable inert collection bag is attached to the expiratory side of a non-rebreathing valve with a resistor in place. Five breaths during relaxed tidal breathing will be sampled, and FeNO will be analysed within 4 hours using a chemiluminescence analyser.

Throat swab
Airway and oral bacteria are metabolically active and may add to the exhaled volatile metabolites. Therefore, airway cultures need to be assessed in all children. Throat swab: a small cotton will be used to swab the posterior nasopharynx and then placed into the culture medium. In expectorating CF patients a sputum sample will be collected.

Exhaled breath condensate:
EBC are collected by a simple glass cold trap which is placed into a cooling sludge (T = -78.5°C, isopropanol/dry ice) following the guidelines by the ATS/ERS task force [77]. The cold trap is fixated on a table for safety and an exhaust line is applied to the cooling sludge to avoid any possible fumes from the isopropanol. The child breathes through a disposable, sterile mouthpiece into a teflon line connected to the cold trap during 5-15 minutes. The samples are immediately stored at -80°C until up-concentration by lyophilisation and analysis with ultra- high performance liquid chromatography high resolution tandem mass spectrometry (UHPLC-HRMS/MS) at either the Kinderspital Zurich or ETH Zurich.

Allergy testing:
Skin-prick test (SPT) for the 7 most common aeroallergens in Switzerland (*D. pteronissimus*, cat, grass mix, birch, hazelnut, ash, and alder) will be performed in all healthy controls and children with respiratory symptoms ≥2y whom allergy testing has never been performed before. Results from allergy testing for most of the patients with respiratory symptoms is available routine clinical laboratory testing.
SPT is a simple, safe and quick test, providing results within 15-20 minutes. A drop of the allergen (extract) solution is placed on the skin of child comfortably seated on a chair or on the lap of the parent. The skin is then pricked through the drop using the tip of a lancet – this can feel a little sharp but is not painful and should not bleed. In case of sensitization to an allergen it elicits a small, localised allergic response, in the form of a wheal (bump) and flare (redness) at the site of testing, which disappears within 15 minutes. In the standard procedure local antihistamine gel is applied after the test to avoid itching.

5.3 Recruitment and Screening
(swissethics 3b) [69]

Preschool children with chronic respiratory symptoms will be recruited at the outpatient clinic of our department.
For optimal matching of healthy controls we will give additional flyers to the parents of children with chronic respiratory symptoms to inform the classmates or groupmates of their children. Healthy siblings also can be considered as a healthy control. In case of interest from potential healthy controls additional information will be provided by the study doctor. If sufficient number of healthy controls is not reached, healthy children will be recruited from public schools, day care centers, from the public (the choice of the schools and nurseries as well as children's events for public recruitment will be decided later according to our needs).

Recruitment comprises of the following:
- Patient contact, assessment for inclusion and exclusion criteria
- Informed consent and appointment for breath testing
- University Children's Hospital Zurich (ca. 20-60 min):breath testing

Participants will be reimbursed for travel expenses upon request and can withdraw from the study without giving reasons at any time without their decision impacting in any way on their clinical care. Healthy control persons (≤ 18 years) will be recruited from the general population by printed flyers, newspaper advertisements and personal communication. They will follow the same testing regimen as children with respiratory symptoms. For healthy controls besides the breath test spirometry, exhaled NO measurement and sin-prick test will be performed. Prior to the test appointment a questionnaire will be filled-in by parents to assess the respiratory health status of the healthy controls.
Participants will not be subjected to any invasive or distressing test or intervention procedures beyond skin prick testing. Other than sparing about one hour of their time to travel to and from the University Children's Hospital Zurich, having above mentioned tests done, participants will not experience any additional inconveniences. All investigators and participants are fully informed about the study design and no concealment or group allocation is necessary. All measurements will be undertaken by a small set of trained investigators in a standardised fashion. Participant confidentiality is a priority.

5.4 Methods of minimising bias

(STROBE 9; swissethics 2) [6,69]

Selection bias: to reduce selection bias all patients with respiratory symptoms fulfilling the inclusion criteria for one of the work packages are asked to participate in the study. Healthy children will be recruited for the general population.
Loss for follow up may occur in the WP II and WP IV. As these children are followed up in the regular outpatient clinic in the same structure loss for follow up we expect to be not higher than 25 %.
Observer bias: the parents will fill in health questionnaires. Investigators performing the measurements are blinded to the clinical data and questionnaire results.
Sequential testing bias: All of the planned measurements will be performed in random order.
Measurement bias: feasibility study has been performed to reduce the measurement bias, tidal breathing with face mask vs single breath with mouthpiece were tested showing comparable results. The mask with the lowest level of plasticizers has been selected [79].

6. PROJECT POPULATION

(STROBE 6; HRO Annex 2/1.2) [4,6]

As our study approach is exploratory, thus the possibilities for sample size estimation are limited.

WP I 'preschool': 180 preschool children aged 6 months to 4 years will be included in WP 1.

Three groups will be investigated:
- 60 Healthy controls
- 60 Patients with chronic respiratory symptoms (wheezing, cough) and low risk for asthma
- 60Patients with chronic respiratory symptoms (wheezing, cough) and high risk for asthma

WP II 'longitudinal'; prospective longitudinal observational cohort study: For prospective assessment of health outcomes parents of the participants of WP I will be asked to take part in longitudinal follow-up study. Participants will be followed until reaching the age where lung function testing reliably can be conducted (six years). Therefore, the duration of the follow depends on the age at study entry (two to six years). Two follow-up schemes will be offered:

This scheme consists of one follow-up visit at the age six. We aim for a participation of rate 75% (n=135) of the WP I population.
- 45 healthy controls
- 45 patients with chronic respiratory symptoms (wheezing, cough) and low risk for asthma
- 45 patients with chronic respiratory symptoms (wheezing, cough) and high risk for asthma

WP III 'school-age'; prospective cross-sectional exploratory observational matched case control study:
In this case-control study we enrol three groups with sex- and age-matched school-aged children (age 5 - 18 years):
- 30 healthy controls
- 30 children with known allergic asthma
- 30 children with known non-allergic asthma

WP IV: 'school-age' assessment during asthma worsening.
Patients from WP III will be asked to return for two additional visits in the case of an asthma worsening. Visit Ex1 will be done within 1 week of start of asthma worsening and Visit Ex2 one month after asthma worsening is resolved. Patients with the need of an acute treatment with systemic corticosteroids or hospitalization will not be included in this part.
We aim to include 10-15 asthmatic patients from WP lll

6.1 Inclusion criteria

All WP:
- informed consent by parents (and by children if age > 8 years)
- active/passive understanding of German or other languages spoken in Switzerland
- No chronic respiratory symptoms or diseases in healthy controls

WP I and II:
All groups:
- age 6 months -4 years

Wheeze group:
- preschool wheeze (more than two episodes of wheeze during 12 months prior to inclusion)

Cough group:
- daily cough for at least 4 weeks prior to inclusion

WP III:
All groups:

- age 5 years - < 18 years

Asthma group:

- doctor diagnosed asthma (according to current guidelines)

6.2 Exclusion criteria

ll WP:

- heart failure diagnosed after birth affecting pulmonary circulation
- specific respiratory diseases: cystic fibrosis; primary ciliary dyskinesia; interstitial lung disease; airway malformation
- any disability that prevents proper execution of any measurement manoeuvre (breath analysis, spirometry)
- fever of at least 38.5°C during the last two weeks prior to the planned first visit

WP III/lV:
Asthma group:

- uncontrolled asthma (assessed by ACQ) requiring oral corticosteroids

6.3 Criteria for withdrawal / discontinuation of participants

Patients need to discontinue from the project in the case of withdrawal of informed consent or if families move to another country. The person who is responsible for anonymization of the data will find and prohibit the data from further analysis.
In case if a healthy control develops chronic respiratory symptoms during the period of longitudinal control, the subject will be withdrawn from the group of healthy controls and in case of fulfilling the criteria of one of the study groups will be offered to continue the follow up as a child with respiratory symptoms.

PROJECT ASSESSMENTS

(STROBE 7; HRO Annex 2/1.2) [4,6]
Describe procedures, measurements, collection, storage of samples taken, data storage, etc.

6.4 Project flow chart(s) / table of procedures and assessments

See above the schedule of assessment (FLOW OF RESEARCH PROJECT).

6.5 Assessments of primary endpoint/outcome

Breath analysis

Prior to the measurement, participants will need to have abstained for at least one hour from smoking, eating (including chewing gum), beverages (except for plain water), brushing of teeth and using mouthwash. This should cause minimal discomfort, as participants are free to indulge in normal daily activities and dietary habits up to one hour before the beginning of the test session and immediately after its completion.

SESI-MS measurement:

- Children ≤4 years of age: for the breath analysis children are comfortably seated on a chair or on the lap of the parent and will be asked to breathe calmly as they usually breathe for about 15 seconds through a sterile mask into the mass spectrometer.

- Infants <1.5 years of age: measurements will be performed while the child is in natural sleep. The child will be in supine position either on the lap of the mother or in the cot. The face mask will be

carefully placed over the nose and mouth of the sleeping child. This procedure is entirely risk-free as no alteration of normal breathing is required. After 6 breath cycles of 15 seconds over a period of several minutes, measurement is complete.

- Children ≥4 years of age: measurements will be performed via a mouthpiece. Comfortably seated participants will be asked to exhale calmly for about 15 seconds through a sterile plastic mouth piece into the MS. This procedure is entirely risk-free as no alteration of normal breathing is required – it is less demanding than routine spirometric testing. After six repeated exhalations over a period of several minutes, testing is complete.

The whole procedure will take 40 minutes.

6.6 Assessment of secondary endpoint/outcome(s)

Individual clinical history for children with respiratory symptoms and questionnaires for healthy controls:

- Children with respiratory symptoms will be characterized by their individual clinical history, including frequency and severity of exacerbations. Demographics, lifestyle and anthropomorphic characteristics will be extracted from clinical records and used in coded form for analyses. Recorded data include age, sex, ethnicity, height, weight, smoking status, comorbidities and medications, and occupation in older children. The following questionnaires and tools will be applied to assess the inclusion/exclusion criteria and the status of the patients: Asthma control and respiratory symptoms: Asthma Predictive Index (API), Paediatric Asthma Quality of Life Questionnaire (PAQLQ), Chronic Cough Quality of Life (CC-QoL), Parent Proxy Chronic Cough Quality of Life (PC-QoL).
- For the healthy controls the LuftiBus questionnaires will be applied in order to collect data on: 1. Comorbidities, personal and family history regard, environmental exposures, socioeconomic status. 2. To assess the respiratory health status (KEK-ZH-Nr. 2014-0491). This questionnaire consists of a general section where subject characteristics, such as age, origin and family, a section on the health behaviour with questions on physical activity and active smoking, a section on respiratory health including questions on colds and allergies, sleep, cough, wheezing, including asthma treatment, and a section on family history and passive smoke exposure.

Lung function (Spirometry)

Lung function will be measured in all children ≥4 years of age. In the case of a recent lung function measurement (less than 2 weeks prior to the study date) no spirometry will be performed. This is a standard procedure routinely used in medical praxis to assess the lung function. The children are asked to take the deepest breath they can, and then exhale into the lung function mouthpiece as hard as possible, for as long as possible, preferably at least 6 seconds. During the test, soft nose clips may be used to prevent air escaping through the nose. Sterile single use filter mouthpieces are used to prevent the spread of microorganisms.
The following parameters will be recorded: forced vital capacity (FVC), forced expiratory volume in first second (FEV1), peak expiratory flow (PEF), FEV1/FVC ratio, maximum expiratory flow at 75%, 50% and at 25% FVC (MEF75, MEF50, MEF25) and MEF75–25.

Fractional exhaled Nitric oxide (FeNO):

The fraction of exhaled nitric oxide (FeNO) is a non-invasive biomarker associated with airway inflammation and can be measured across all ages in standardized fashion. For the reservoir-offline technique, children are sitting on the legs of the parent or a chair while tidally breathing NO-free air for ten breaths. A reusable inert collection bag is attached to the expiratory side of a non-rebreathing valve with a resistor in place. Five breaths during relaxed tidal breathing will be sampled, and FeNO will be analysed within 4 hours using a chemiluminescence analyser. FeNO will be assessed in healthy controls only.

Allergy testing:
Airway allergies can result in significant airway inflammation. Therefore, the allergy status is important to know. Skin-prick test (SPT) for the 7 most common aeroallergens in Switzerland (D. pteronissimus,

cat, grass mix, birch, hazelnut, ash, alder) will be performed in all healthy controls. Results from allergy testing is available for all CF patients from routine clinical laboratory testing (Jahreskontrolle).
SPT is a simple, safe and quick test, providing results within 15-20 minutes. A drop of the allergen (extract) solution is placed on the skin of child comfortably seated on a chair or on the lap of the parent. The skin is then pricked through the drop using the tip of a lancet – this can feel a little sharp but is not painful and should not bleed. In case of sensitization to an allergen it elicits a small, localised allergic response, in the form of a wheal (bump) and flare (redness) at the site of testing, which disappears within 15 minutes. In the standard procedure local antihistamine gel is applied after the test to avoid itching.

Airway bacteriology
Airway and oral bacteria are metabolically active and may add to the exhaled volatile metabolites. Therefore, airway cultures need to be assessed in all children. Throat swab: two small cotton swabs will be contemporaneously used to swab the posterior nasopharynx. One will be immediately placed into the culture medium and the other one will be frozen at -80°C for further analyses (microbiome).

Exhaled breath condensate:
EBC are collected by a simple glass cold trap which is placed into a cooling sludge (T = -78.5°C, isopropanol/dry ice) following the guidelines by the ATS/ERS task force [77]. The cold trap is fixated on a table for safety and an exhaust line is applied to the cooling sludge to avoid any possible fumes from the isopropanol. The child breathes through a disposable, sterile mouthpiece into a teflon line connected to the cold trap during 5-15 minutes. The samples are immediately stored at -80°C until up-concentration by lyophilisation and analysis with ultra- high performance liquid chromatography high resolution tandem mass spectrometry (UHPLC-HRMS/MS) at either the Kinderspital Zurich or ETH Zurich.

Venous blood samples (only for children with respiratory symptoms) will be obtained during patients' routine outpatient visits if clinically indicated. The aim is to assess specific and total IgE and certain food metabolites such as fatty acids. 500µl of blood will be used to assess the total blood count. In addition, 5ml of blood shall be stored fin the CK-Care Biobank for later analyses, such as new specific nutrients and genetic analyses. Before punctuation of the child's skin EMLA® cream will be used for local anaesthetic purpose.

With their informed consent, patients with chronic respiratory symptoms agree to the strictly coded use of their confidential clinical records held by the University Children's Hospital of Zurich in present and future research purposes. One of the investigators will be responsible for extracting relevant details from the registry followed by removal of any identifying information for further analysis. Participants will be informed that other than the investigators, only authorised staff of the responsible ethics committee are allowed access to confidential information for auditing and monitoring purposes under strict adherence to patient confidentiality.

6.7 Assessment of other study variables

We designed a CRF (Clinical report form) to assess all above mentioned confounders, effect modifiers and exposers together with primary and secondary endpoints.

6.8 Assessment of safety and reporting
(HRO Art. 20. 21) [4]

This project is categorized as a non-clinical research project, risk category B.

Participants will not be subjected to any invasive or distressing test or intervention procedures beyond routine clinical management plan. Other than sparing about one hour of their time to travel to and from the Kinderspital, blowing through a sterile mouthpiece and mask, participants will not experience any additional inconveniences. All investigators and participants are fully informed about the study design and no concealment or group allocation is necessary. All measurements will be undertaken by a small set of trained investigators in a standardised fashion. Participant confidentiality is a priority.

No adverse events are expected to arise from this study.

All measurements will be supervised by medically trained investigators. All participant contact will take place in the Kinderspital, guaranteeing easy and quick access to emergency alert systems and resuscitation equipment.

The primary outcome – breath analysis – involves a single, 20 min session that asks comfortably-seated participants to exhale calmly into a plastic mouth piece and mask connected to the mass spectrometer. Participants exhale six times interspersed with adequate rest periods without excessive force into the SESI-MS. They are not exposed to any environmental hazards like radiation or chemical vapours.

Thus, breath sampling is a non-invasive, harmless, effortless procedure that is not expected to entail any distress other than the minor inconvenience of traveling to the Kinderspital Zurich.

Only procedures with minimal risk and burden (questionnaire, breath collection, lung function, throat and nasal swaps, sputum collection and skin prick testing) are applied. Healthy controls may experience some itching after skin prick test (usually up to 15 minutes) in case of having sensitization to an allergen. As a routine procedure, local antihistamine gel will be applied on that area of skin to relief and decrease the duration of itching.
Extremely rarely allergic reactions can occur within the the skin prick test, if a previously unknown severe allergy is present. This can occur because a droplet of solution containing an allergen is used during the test. These allergic reactions are mainly described in the testing of food or insect allergies (wasps and bees). The frequency is within the scope of 1-4 examinations per 10'000 tests. We only examine inhalant allergens (such as grasses, tree pollen, house dust mites and animal hair). In these allergens, reactions are much rarer. If a relevant allergic reaction still occurs, this can be treated immediately with available medicines.

6.8.1 Definition of Serious Events (SEs)

(HRO Art. 21) [4]

A **serious event** is any unfavourable event for which a causal relationship to sampling of biological material or the collection of health related personal data cannot be ruled out, and which:

- requires hospitalisation or prolongation of an inpatients' hospitalisation,
- results in persistent or significant disability or incapacity, or
- is life-threatening or results in death,

Due to the nature of the study and related study procedures no serious events are expected.

6.8.2 Assessment and Documentation of SEs

(HRO Art. 20, 21) [4]
The assessment by the project leader with regard to the project-specific measure relation is done according to the following definitions:
Unrelated: The occurrence of the event has no temporal relationship to the project-specific measures applied and can be explained by the underlying disease or other factors.
Related: There is a plausible temporal relationship between the occurrence of the event and the project-specific, applied measures and cannot be explained by the underlying disease or other factors.

All SEs are to be documented in the participants´ file and on the SE report form.

6.8.3 Reporting of SEs, Safety and Protective Measures

(HRO Art. 20) [4]
The project leader shall report any occurring SE to the responsible EC within 7 days (and to the FOPH in case of involved radioactive sources). He/she shall also submit a report which evaluates the relationship between the event reported and the methods of collecting health related personal data or sampling of biological material within that project, furthermore proposals how to proceed with the project.
The project leader shall notify the EC within 7 days of any immediate other safety and protective measures, which have to be taken during the conduct of the research project. In addition, the project leader shall explain the circumstances, which necessitated the safety and protective measures.

7. STATISTICAL METHODOLOGY

7.1 Determination of Sample Size

(STROBE 10) [6]

Traditional methods for estimating sample sizes needed to detect a minimum meaningful difference cannot be applied to our exploratory pilot design. Tentative estimations can be made based on sample size requirements for factor analysis [73]: Assuming a variable-to-factor ratio of at least seven, the minimum necessary sample size for good (92%) model agreement is 55 to 80. Calculations for proteomic profiling using MS [74] suggest a similar range of sample sizes. Based on our prior experience of exploratory breath analysis [52, 55] we aim to recruit at least 60 patients with chronic respiratory symptoms with high risk for asthma, 60 patients with chronic respiratory symptoms with low risk for asthma and 60 healthy controls for WP I. We assume that we can recruit 75% of the original population to WP III, 30 allergic asthma patients, 30 no allergic asthma patients and 30 healthy controls for WP II.

7.2 Data processing

Paper documents and informed consent forms will be stored in a lock-secured cupboard in a dedicated research office at the Division of Pulmonology, University Children's Hospital of Zurich. Only study investigators have access to these documents. Authorised staff of the responsible ethics committee can request access to these data for monitoring and auditing purposes.

Electronic data will be stored in a password-secured coded spreadsheet file. The file will be stored within the Division of Pulmonology section of the University Children's Hospital of Zurich intranet server. It can only be accessed by authorised users from registered computers at the University Hospital of Zurich.

In the current study, exploratory comparisons between patients with chronic respiratory symptoms and healthy controls' breathprints shall be undertaken through statistical data reduction techniques. As previously demonstrated, this approach can drastically limit the number of compounds-of-interest – a crucial necessity for translating breathprint analysis into day-to-day clinical care [76].

7.3 Planned analysis

(STROBE 12a-d) [6]

Demographic and clinical characteristics will be compared using independent-sample pairwise analysis methods (i.e. ANOVA, t-tests, Mann-Whitney U-tests) with Bonferroni-correction for multiple comparisons. Exhaled breath composition will be evaluated through a stepwise application of statistical data reduction approaches. Bonferroni-corrected, non-parametric one-way analyses of variance (Kruskal-Wallis tests) will be employed to select significant discriminatory mass-to-charge (*m/z*) peaks. Thereafter, principle component analysis, multivariate analysis of variance and canonical analysis will be used to extract meaningful summary statistics. Data analysis will be done with Matlab (R2016b and newer, The MathWorks Inc., Natick, MA, US) and R (Development Core Team, R Foundation for Statistical Computing, Vienna, Austria) based on data analysis tools developed at ETH Zurich.
The SESI-MS data will be acquired and mass calibrated with the Analyst TF 1.7 and PeakView 2.1 software (Applied Biosystems Sciex, Toronto, ON, Canada).

7.3.1 Datasets to be analysed

The final dataset will include patient characteristics, clinical data, and data from the questionnaires and the selected features from SESI-analysis.

7.3.2 Handling of missing data

As the study is depending on successful SESI-measurements, we will recruit further patients and controls to replace / compensate missing data and drop-outs for primary outcomes. Missing data from secondary outcomes will be acknowledged accordingly in the statistical analyses.

7.3.3 Ancillary analysis

Ancillary analyses will be planned based on the results from the primary statistics.

7.3.4 Deviations from the original statistical plan

The mathematical and statistical development in the analysis of large, multidimensional datasets is highly dynamic and improvement of the available methods are likely to occur in the near future. As we

closely collaborate with the ETH department of Computer Science, our data will be analysed state-of-art and any deviation from the planned analyses will be reported in the respective scientific articles.

8. DATA AND QUALITY MANAGEMENT

(HRO Art. 5, 25-27, Annex 2/1.7) [4]

8.1 Data handling and record keeping / archiving

(HRO Art. 5, 26, 27, Annex 2/1.7)

Paper documents and informed consent forms will be stored in a lock-secured cupboard in a dedicated research office (SP.C06) at the Division of Respiratory Medicine, University Children's Hospital of Zurich. Only study investigators have access to these documents. Authorised staff of the responsible ethics committee can request access to these data for monitoring and auditing purposes.
Electronic data will be stored in a password-secured coded spreadsheet file. The file will be stored within the Division of Pulmonology section of the University Children's Hospital of Zurich intranet server. It can only be accessed by authorised users from registered computers at the University Children's Hospital of Zurich.

8.2 Confidentiality, Data Protection

(HRO Art. 5) [4]

Data generation, transmission, storage and analysis of health related personal data and the storage of biological samples within this project will follow strictly the current Swiss legal requirements for data protection and will be performed according to the Ordinance HRO Art. 5.
Health related personal data captured during this project and biological samples from participants are strictly confidential and disclosure to third parties is prohibited; coding will safeguard participants' confidentiality.
Data protection: project data shall be handled with uttermost discretion and only be accessible to authorised personnel. Include a statement that direct access to source documents will be permitted for purposes of monitoring, audits or inspections and should declare who will have access to project plan, dataset, statistical code, etc. during and after the research project (publication, dissemination).
Strict participant confidentiality in accordance with established best clinical practice and Swiss legal requirements will be adhered to88. Only medically trained investigators will have access to clinical records. Once consented, all participant data used for analysis will be stored in a coded and secure format. Each participant will receive a code of the study location (e. g. continuous number according to time of enrolment continued by letters identifying the health status (CF or healthy control) and finished by a planned date of investigation). Patient details will be anonymous to anybody except authorised study investigators. A password secured electronic file only accessible by the principle investigator and his designee will act as the only link between the participant code and his or her real name, contact information and medical/study related data. Individual participants will not be identifiable from the results as submitted for publication. Data analysis will only use coded data records. Patients agree to confidential, coded use of information contained in their clinical records for research purposes as outlined in this protocol. Participants are informed that authorised staff of the responsible ethics committee of the Canton of Zurich, Switzerland, can obtain access to confidential information for monitoring and auditing purposes under strict adherence to confidentiality rules.

8.3 Coding

(HRO Art. 25-27) [4]

Each participant will receive a code of the study location (e. g. continuous number according to time of enrolment continued by letters identifying the health status (child with chronic respiratory symptoms or healthy control) and finished by a planned date of investigation). Patient details will be anonymous to anybody except authorised study investigators.

8.4 Archiving and Destruction

All data will be stored for 10 years on password secured databases with backup files within the Kinderspital. Hardcopies will be stored for 10 years after completion of the study in a specific locked room with access only for the investigator. Stored biomaterial will be destroyed according to the hospital guidelines by the clinical chemistry laboratory 5 years after completion of the study.

9. PUBLICATION AND DISSEMINATION POLICY

(HRO Art. 15j; STROBE 22; HRO Annex 2/1.10) [4,6]

9.1 Publication of results

Enrolees can indicate during the consent procedure if and how they would like their individual results to be communicated. Once data collection and analysis are complete, participants who have indicated their desire to be informed of the overall study findings will be contacted by investigators to disclose the overall study findings as prepared for publication.

Study results will be communicated to the public through publication in peer-reviewed scientific media. In addition results that are interesting for the public will be summarized in lay language and presented within study specific newsletters.

Dissemination of results will be independent of negative or positive findings and submission of results for publication will follow the consensus decision of all investigators.

Results will be presented at national and international conferences in abstract form.

Authorship will be handled according to current recommendations by the “Akademien der Wissenschaften Schweiz“: Autorschaft bei wissenschaftlichen Publikationen; 2013

9.2 Data sharing

There is no data sharing planned. If a third party requests the data for analysis this will be discussed among the investigators and, if agreed on, only anonymised data will be provided.

10. FUNDING AND SUPPORT

(HRO Art. 15j; STROBE 22; HRO Annex 2/1.10) [4,6]

Several funding sources are currently involved in the project: Long term research fellowship from the European Respiratory Society (total 70’000) and from private donations (total of 240’000.-) Research grant from UZH foundation: (170’000). We plan an application to the Swiss National research foundation.

We declare that none of the members from this study has any intellectual or financial conflict of interest.

11. INSURANCE

(HRO Annex 1; HRO Annex 2/1.6) [4]

Insurance is covered by the Haftplichtversicherung “Zurich Versicherung“ by PD Dr. med. Alexander Möller and the Kinderspital. This policy covers any damage or adverse consequence incurred through participation in this study. Participants` insurance coverage is strictly bound to their following of

instructions from study personnel. Investigators must be instantly informed of the occurrence of any damages or adverse events related to participation in the study.

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13. APPENDICES

Add any appendices here if relevant. For documents that change very frequently consider mentioning as separately provided documents with a link included here.