Study protocol version 3.0, 5th October 2019.

Research Study: ***BIOCROSS [BIOmarkers in CROSS-sectional study]- Influence of constitutive and disease-related factors on levels of non- invasive biomarkers in subjects with asthma and other respiratory diseases at baseline and during follow-up of treatments***

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1. Aim

The overall goal of this study is to measure baseline levels of biomarkers in well characterized and phenotyped subjects in order to establish profiles of biomarkers that are specific for different sub-phenotypes of the investigated diseases. The focus of the project is to find biomarkers that may be assessed by non-invasive methods at the point-of-care and used in precision-medicine efforts to select treatments. It is therefore planned to collect and analyse biomarkers recovered in the non-invasive matrices urine, blood, saliva and e:xhaled air. The goal is to find measures that may replace invasive examinations for diagnostic purposes, for early detection of exacerbations and deterioration of disease, and monitoring of treatment.

The biomarker candidates will mainly be measured by the use of high-tech analytical platforms for lipidomics (prostaglandins, leukotrienes and related lipid mediators, phospholipids, sphingolipids and surfactants), and proteomics. Breathomics is one particular novel approach of the project which aim to identify also VOCs (Volatile Organic Compounds) and particle-associated biomarkers in the exhaled air. Data will be related to standard clinical tests and established general biomarkers such as white blood cell counts, CRP and specific IgE.

2. Rationale and Hypothesis

Asthma is a heterogeneous disease with several different underlying phenotypes. Although patients have similar symptoms and the same diagnosis - asthma, treatment must be individualised because one targeted treatment may have remarkable effects in specific preselected subgroups of patients, but may be useless and side-effect prone in others.

The presence of different sub-phenotypes seems to exist also in other respiratory diseases. Indeed, symptoms such as breathlessness, cough and increased sputum may be caused by asthma, COPD or other respiratory afflictions including sarcoidosis, bronchiectasis and lung-fibrosis. Common symptoms of respiratory diseases are among the top five causes of visits in primary care both for adults and children, but there is currently no single golden standard test that quickly and reliably can provide the correct diagnosis and determine choice of treatment. Patients are therefore put through a multitude of examinations that include a combination of patient history, lung function measurements, x-ray, computer tomography, exclusion of alternative diseases, and often also a trial period with symptomatic medications.

Taken together, there is a great need for new predictive biomarkers that can improve diagnosis of asthma, establish phenotype-differences and differentiate asthma from other diseases of the lung and the airways.

The current study is part of the ChAMP project which in turn builds on previous clinical and epidemiological cohort studies such as BIOAIR, U-BIOPRED and Swedish Ga2len, all being supported in part by EU or IMI grants. The project has access to cutting-edge new methods for molecular phenotyping and now wish to extend and validate findings done in the previous studies by comparing biomarker measurements previously primarily done in asthma or COPD with data from patients with other lung diseases. Previous work suggests not only differences between patient groups with respect to biomarkers according to per protocol clinical recruitment group but also that new, and presumably pathobiologically connected, dusters of patients may be identified according to molecular fingerprints. In order to permit comparisons with previous studies, the healthy subjects and patients with asthma and COPD have been recruited to meet the criteria of in particular the U-BIOPRED investigation. In addition, data from this study may be part of the new IMI project 3TR where biomarkers of treatment responsiveness will

be studied across seven immunemediated diseases (RA, SLE, MS, Mb Crohn, Ulcerative Colitis, COPD and asthma).

This cross-sectional study therefore rests on the operational hypothesis that measurements of biomarker profiles in non-invasive samples from clinically well characterised patients with asthma will permit both distinction from COPD and other respiratory diseases. In addition, it is proposed that such measurements and their integration using systems biology strategies that are part of the ChAMP project will aid the identification of sub-phenotypes that relate to specific disease-driving mechanisms and therefore may govern selection of new personalised treatments.

3. Design

The project has two parts:
a) A cross-sectional collection of samples for biomarker research in healthy controls and in clinically well characterized and phenotyped patients belonging to different disease-groups as seen in the every-day clinic.
b) A longitudinal follow-up of subgroups of patients with similar examinations and sampling for biomarkers as at baseline. All patients in the severe asthma group being treated with biological therapies will be followed for up to three years.

Patients will be recruited to include asthma of different severities and phenotypes, COPD stages II-III, sarcoidosis, subjects with bronchiectasis and subjects with interstitial lung diseases (both idiopathic pulmonary fibrosis and non-specific interstitial lesions). The intention is to recruit healthy control subjects who are age- and gender- matched because many biomarker candidates display relations to sex and age. The healthy controls and the asthma groups will be selected using the same inclusion and exclusion criteria as in the IMI study U-BIOPRED [Shaw et al Eur Respir J. 2015;46:1308-21] in order to facilitate comparisons. The bronchiectasis patients will be included in accordance with the EMBARC study ([EPN-2016 / 164 7-31/2], McDonnell MJ et al, Lancet Respir Med. 2016;4:969-979.). The subjects in the sarcoid group and subjects with interstitial lung diseases will be diagnosed and included according to internationally established clinical criteria.

4. Subjects

4.1 *Source of subjects*

All patient cohorts (non-smoking subjects with asthma of different severities and phenotypes, subjects with COPD stages II-III, sarcoidosis, bronchiectasis and interstitial lung diseases), will be recruited from the existing patient cohorts at the Lung and Allergy clinic at the Karolinska University Hospital, as well as from the other participating centres. Subjects with asthma and healthy controls will in addition be recruited from the GA^{2}LEN-cohort in Sweden which altogether includes around 1300 patients with asthma and healthy controls. Suitable participants will be identified from patient records or existing registries by members of the study team. Patients with lung fibrosis will be also recruited from the Swedish National Pulmonary fibrosis registry.

Patients will be approached at the time of a routine clinic visit and invited to participate. Written information regarding the study (the patient information sheet) and contact details of the study team will be given. Subjects interested in participating in the study will be invited to attend a screening visit.

The healthy controls will also be recruited by the use of advertisements. A contact number will be given and further information regarding the study will be provided. Interested participants will be sent a copy of the patient information sheet and invited to attend the screening visit.

Written informed consent will be obtained by the study team at the time of the screening visit -prior to undertaking any study procedures. "Informed consent" requires individual discussion with the subject about the study and the nature of the procedures to be conducted in a language that is easy to comprehend. The subject should understand that his / her refusal to participate in the study will not affect their subsequent medical care and if they do consent they may withdraw at any point without affecting their care.

The subject consent form must be signed by a member of the study team to confirm that informed consent was obtained. The original of the signed declaration of consent should be stored in the case notes. A copy should be given to the subject.

4.2 *Number of subjects and power calculation*

The clinical and biomarker measurements will be analysed using appropriate and validated standard statistical methods, including multivariate analysis. Concerning the sample size, a total of up to 900 subjects will be recruited and enrolled into the seven cohorts (described below) at the participating centres in Sweden.

One main aim with the current project is to discover new biomarkers in samples collected from the investigated cohorts of well-characterised patients (including exhaled air) and to discover new mechanisms of respiratory diseases. There are no previous studies with the current methodology in patients with severe airway disease. It is however known that meaningful and statistically significant differences with respect to clinical measurements, questionnaire data and primary biomarkers such as lipid mediators in urine, blood and cells in sputum, have been found in previous asthma studies we have performed after inclusion of even less than 100 subjects in each recruitment group. Because several of the biomarkers to be studied represent explorative cutting edge science where nothing is known about variability in these particular patients, it was decided to nevertheless aim for the least 100 number of patients to be included in each study group in the BIOCROSS study. Furthermore, the investigators responsible for biochemical and molecular measurements have extensive experience from other studies such as BIOAIR, U-BIOPRED and Swedish GA^{2}LEN. In addition, in this project, interim analysis will be performed when 50 subjects have been included in each study group in order to further address the power issue.

4.3 *Subject cohorts*

A total of up to 900 subjects (males and females aged 18 years or older) will be recruited and enrolled into the 7 cohorts over the study period.

Cohort 1 Severe Asthma: Up to 200 severe asthmatics on high dose ICS and /or OCS; non-smokers, ex-smokers and current smokers *(=U-BIOPRED Cohorts A and B)* can be included (see section 4.4). Eosinophilic exacerbation prone phenotype to be treated with anti-IL 5, anti-IgE and other biologics will forma subgroup of Cohort 1.

Cohort 2 Mild* to *moderate Asthma: Up to 100 mild to moderate asthmatics on regular inhaled corticosteroids (ICS); non-smokers. *(=U-BIOPRED Cohort C)*

***Cohort* 3 COPD:** Up to 100 subjects with COPD according to GOLD severity classification II-III, ex-smokers.

Cohort 4 Bronchiectasis: Up to 100 subjects diagnosed according to EMBARC criteria.

***Cohort 5* Sarcoidosis:** Up to 100 subjects diagnosed according to established clinical criteria.

***Cohort* 6 *Interstitial lung* disease:** Up to 100 subjects diagnosed according to established clinical criteria.

Cohort 7 Healthy volunteers: Up to 200; non-smokers with pre bronchodilator FEVl> 80% predicted *(=U-BIOPRED Cohort D):*

4.4 Definition of smoking history

Definition of non-smokers:
A non-smoker for at least the past 12 months with a pack history of less than 5 years.
Definition of an ex-smoker: A non-smoker for at least the past 12 months with a pack history of more than 5 years
Pack years = No of cigarettes smoked / day x No of years smoked
Definition of smokers: Current smoker and pack history of more than 5 years

5. Inclusion and exclusion criteria

5.1 General inclusion criteria - all cohorts

A subject will be eligible for inclusion in this study only if all of the following criteria apply:

1. Able to give written informed consent prior to participation in the study, which includes ability to comply with the requirements and restrictions listed in the consent form. lnformed consent must be obtained prior to undertaking any study procedures.
2. Male or female subject aged 18 years or older at screening.
3 Able to complete the study and all measurements.
4. Able to read, comprehend, and write at a sufficient level to complete study related materials.

5.2 General exclusion criteria - all cohorts

A subject will not be eligible for inclusion in this study if any of the following criteria apply:

1. As a result of medical interview, physical examination or screening investigation the physician responsible considers the subject unfit for the study either because of the risk to the subject due to the study or the influence this may have on the study results.
2. The subject hasa history of recreational drug use or other allergy, which, in the opinion of the responsible physician, contra-indicates their participation.
3. Subject is female who is pregnant or lactating or up to 6 weeks post partum or 6 weeks cessation of breast feeding. If a woman is subsequently found to have been pregnant at the time of an assessment data from that assessment will not be included in the analyses.
4. Those who, in the opinion of the investigator, have arisk of non-compliance with study procedures.
5. The subject hasa recent history of incapacitating psychiatric disorders
6. History or current evidence of an upper or lower respiratory infection or symptoms (including common cold) within 4 weeks of baseline assessments (assessments should be deferred).
7. Treatment with antibiotics and/ or oral corticosteroids <luring the past 4 weeks, unless prescribed as maintenance medications.
8. Severe comorbidity (eg underlying cystic fibrosis, coronary artery disease, heart failure).
9. Respiratory insufficiency and need for home oxygen therapy
10. Any subjects currently participating, or having participated within 3 months of the last dose in a study using a new molecular entity, or any other study considered to interfere with the objectives of the current study.

5.3 lnclusion criteria Cohort 1 and 2: Asthma

Patients who have asthma diagnosis after investigation and assessment at specialist

clinic and fulfil the general exclusion and inclusion criteria and fulfil other special exclusion and inclusion criteria according 5.3.1 and 5.3.2 could be included.

5.3.1 Additional inclusion criteria for the severe asthma, cohort 1

1. Subjects must have been under the follow up of a respiratory specialist for at least 6 months prior to enrolment in the study. During this time appropriate investigations should have been performed to exclude other diagnoses and steps taken to optimise asthma control including treatment of co-morbidities, assessment of adherence, and reduction in allergen exposure in sensitised subjects
2. Subjects must be uncontrolled (according to GINA guidelines, see below) and/ orhave frequent severe exacerbations (2 per year) despite the prescription of high dose inhaled corticosteroids (. 1000 µg FP daily or equivalent) or maintenance oral corticosteroids plus a long acting beta agonist or one other controller medication (for example anti-cholinergics, leukotriene receptor antagonists or theophylline).

Definition of uncontrolled asthma: three or more of the following features present in any week in the previous 4 weeks:

- o Daytime symptoms more than twice per week
- o Any limitation of activities
- o Nocturnal symptoms once or more per week
- o Need for reliever treatment more than twice per week
- o Pre bronchodilator FEV1 <80% predicted or personal best

OR

- o Frequent severe exacerbations (more than 2 per year; requiring high dose OCS or a doubling of maintenance OCS for at least 3 days),

OR

- o Require prescription of daily or alternate day oral corticosteroids (OCS) to achieve asthma control according to ACT or ACQ

3. Ex-smokers and current smokers *(U-BIOPRED group* B) will be considered a subgroup in data analysis. The same applies to subjects receiving treatment with new biologics on clinical grounds despite not fulfilling all inclucion or exclusion criteria. For primary data analysis, they will be kept as a separate subgroup.

5.3.2 Additional exclusion criteria for the severe asthma, cohort 1

1. Subject has changed asthma medication within the 4 weeks prior to screening (except those using the Symbicort maintenance and reliever therapy.
2. Subject had a severe asthma exacerbation (requiring high dose OCS or a doubling of maintenance OCS for at least 3 days) in the previous month.
3. Significant alternative diagnoses that may mimic or complicate asthma, in particular dysfunctional breathing, panic attacks, and overt psychosocial problems (if these are thought to be the *major* problem rather than *in addition* to severe asthma)
4. Significant other primary pulmonary disorders in particular pulmonary embolism, pulmonary hypertension, interstitial lung disease and lung cancer
5. Subjects with emphysema and bronchiectasis should only be excluded if this is thought to be the major pulmonary disorder rather than *in addition* to severe asthma
6. Diagnosis or current investigation of occupational asthma
7. Diagnosis of chronic inflammatory diseases other than asthma (inflammatory bowel disease, rheumatoid arthritis)

5.3.3 Additional inclusion criteria for the mild to moderate asthma, cohort 2

1. A non-smoker for at least the past 12 months with a history 5 pack years.
2. Subjects must be controlled or partially controlled (according to GINA guidelines as defined below) on a dose of ICS of :,;500 µg FP daily or equivalent without the need for other controller medications (anti-cholinergic, leukotriene receptor antagonists or theophylline) than long acting :fS2 agonists.

Definition of controlled asthma: all of the following must apply in the previous 4 weeks:

- Daytime symptoms twice per week or less
- No limitations to activities
- No nocturnal symptoms
- Need for reliever treatment twice per week or less
- Pre bronchodilator FEV_1 80% predicted

Definition of partially controlled asthma: one or two of the following measures present in any week in the previous 4 weeks:

- Daytime symptoms more than twice per week
- Any !imitations to activities
- Any nocturnal symptoms
- Need for reliever treatment twice per week or more
- Pre bronchodilator FEV_1 < 80% predicted

5.3.4 Additional exclusion criteria for the mild to moderate asthma cohort 2

Same as severe asthma, 5.3.2 as applicable.

5.4 Inclusion criteria Cohort 3: COPD

1. An ex-smoker and a smoking history of more than 15 pack years.
2. The patient has no history of asthma.
3. Subjects must fulfil the general exclusion and inclusion criteria and specific criteria for this cohort.

Specific criteria for this cohort:

The patient has COPD according GOLD-diagnostic criteria FEVif FVC ratio of less than 70%, stage II-III (post bronchodilator FEV_1 < 80% predicted but more than 30% of predicted).

5.5 Inclusion criteria Cohort 4: Non-cystic fibrosis bronchiectasis (NCFB)

1. A non-smoker or ex-smoker.
2. Subjects must fulfil the general exclusion and inclusion criteria and the specific criteria for this cohort.

Specific criteria for this cohort:

Patients who have chest HRCT scan confi.rming the presence of bronchiectasis lesions and have symptoms of chronic productive cough (according to inclusion and exclusion criteria in the EMBARC study ([EPN-2016/1647- 31/2], [12]).

5.6 Inclusion criteria Cohort 5: Sarcoidosis

1. A non-smoker or ex-smaker.
2. Subjects must fulfill the general exclusion and inclusion criteria and the specific criteria for this cohort.

Specific criteria for this cohort:

Patients with pulmonary sarcoidosis will be diagnosed according to criteria defi.ned by World Association of Sarcoidosis and Other Granulomatous disorders (WASOG) [Statement on sarcoidosis. Joint Statement of the American Thoracic Society (ATS), the European Respiratory Society (ERS) and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) adopted by the ATS Board of Directors and by the ERS Executive Committee, February 1999. *American journal of*

respiratory and critical care medicine 1999; 160: 736-755]. The criteria included characteristic symptoms, chest radiography and/ or chest HRCT fi.ndings, typical BAL cell differential counts including an elevated CD4/CD8 ratio, and ruling out other causes of such fi.ndings. There were also positive biopsy fi.ndings (non-necrotizing granulomas) identifi.ed through bronchoscopy with BAL, mediastinoscopy, fine nedle aspiration through bronchi (EBUS) or esophagus (EUS), or from other engaged organs such as lymph nodes or skin. A few patients were diagnosed through decisions at multidisciplinary conferences. Most patients had a chronic disease with disease duration >2 years, and with different stages of disease as defi.ned by chest radiography staging (0, I, II, III, IV).

5.7 Inclusion criteria Cohort 6: Interstitial lung disease

1. A non-smoker or ex-smoker.
2. Subjects must fulfill the general exclusion and inclusion criteria and specific criteria for this cohort.

Specific criteria for this cohort:

Patients who have had the effort dyspnea and/ or symptoms of chronic unproductive cough and chest HRCT scan confirming the presence of interstitial lesions such as UIP. Subjects in this group could have combination of UIP-pattern according HRCT and, if available, surgical lung biopsy pattern, which have been assessed by central reviewers and are consistent with diagnosis of IPF (according to most recent ATS/ERS/JRS/ALAT IPF guideline) and/or have received IPF-diagnoses after discussion/review and decision by multidisciplinary conference.

The patients who fulfil the general exclusion and inclusion criteria, have had symptoms as above, and chest HRCT scan confirming the presence of interstitial lesions which does not fulfil all the criteria for UIP (ie suspected UIP or NSIP-pattern according HRCT) and patients condition is to bad to do lung biopsy could be include in subgroup 6a. ,

5.8 Inclusion criteria Cohort 7 - Healthy volunteers - control cohort *(=U-BIOPRED Cohort D):*

Subjects in the healthy control cohort must fulfil the general exclusion and inclusion criteria and specific criteria for this cohort. This cohort will be recruited from other studies such as Scapis-PeXA-study and Swedish GA2LEN cohorts at the participating centres in Sweden, as well as by advertisements.

5.8.1 Additional inclusion criteria for the healthy control cohort

1. A non-smoker for at least the past 12 months with a pack history 5 pack years.
2. Healthy; defined as individuals who are free of significant cardiovascular, pulmonary, gastrointestinal, hepatic, endocrine, renal, hematological, neurological and psychiatric disease as determined by medical history, physical examination and clinical chemistry/hematology /urinalysis investigation.
3. Pre bronchodilator FEVl ;:;:80% predicted and increase FEV1 < 12% after bronchodilator.

6 Visit structure

All subjects will undergo a screening visit (visit 1) and a study visit (visit 2). No assessment should be taken prior to signature of the consent form. The screening visit should take place within 28 days prior to study visit. At the screening visit the algorithm should be completed (see Appendix 1) to assess eligibility for the study and enrolment to the appropriate cohort. The order of the assessments will be detailed in the standard operating procedures (SOPs) for assessments (Appendix

2,3,4). Training and standardization will occur between sites prior to the study start.

A sub-group of twenty patients from each cohort will attend one additional visit (Visit 3) for repetition of the measurements and the sampling done at visit 2. In addition, all patients in all cohorts to be treated with anti-IL 5, anti-IgE and other biologics will attend visit 3 (4 months after visit 2) and a further one-year follow-up where the measurements and the samplings are repeated once again (visit 4, 12 months after visit 2). The patients who are treated with biologics will also be invited to continued annual follow-ups using the same protocols as during visit 4. The study design for patients on biologics is illustrated in Figure 1 below.

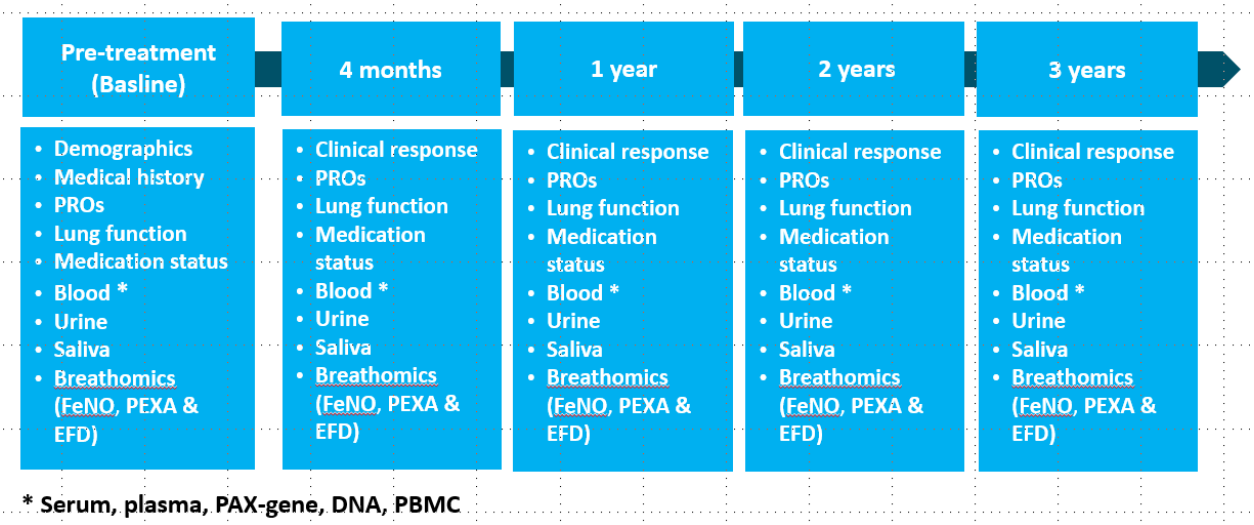


Figure 1: Study design for patients that undergo longitudinal follow-up after the initial BIOCROSS cross-sectional visit.

6.1 Visit 1: Screening visit

a) Information about the study and informed consent (written)
b) Medical history
c) Drug history
d) Current medication
e) Exacerbation history
f) Physical examination
g) Pulse and blood pressure
h) Demography
i) Spirometry and Bronchodilator reversibility[s} J
j) Blood samples for routine laboratory tests and for biomarkers in blood (20 mL in total, see section 7.4)
k) Completion of algorithm for study inclusion and exclusion criteria.
1) Methacholine challenge according to protocol in appendix for 20 healthy Subjects from Healthy control cohort 7. PEXa before and after methacholine.

6.2 Visit 2: Study visit

a) FeNO, IOS, plethysmography, DCLO or SBW (Single Breath N2 Washout), and 6 minute walk test (6MWT for COPD and Interstitial lung disease)
b) Blood samples for routine laboratory tests and baseline samples for biomarkers in blood (90 mL), saliva, urine and exhaled breath (EFD, eNose, PExA).
c) SGRQ for all groups (except healthy controls) and:

- ACQ6 and mini-AQLQ for asthma
- CAT for COPD
- K-BILD for Interstitial lung disease

- QoL-B for bronchiectasis

6.3 Visits 3 and 4: Follow up visits (also annually after visit 4)

Twenty subjects from each of the cohorts will be invited to a follow up visit (visit 3) at 4 months fora sub-study on repeatability.
In addition, all subjects in all cohorts who fulfill criteria for starting treatment with anti-IL 5, anti-IgE or other biologics will be invited to longitudinal assessments within the practice of clinical care at 4 (visit 3) and 12 months (visit 4) after visit 2, and will continue to do this annually for at least three years.

a) FeNO, IOS, plethysmography, DCLO or SBW (Single Breath N2 Washout), and 6MWT
b) Blood samples for routine laboratory tests and samples for biomarkers in blood (90 mL, see section 7.4), saliva, urine and exhaled breath (EFD, eNose, PEXa), IOS.
c) SGRQ for all groups except healthy volunteers and:

- ACQ6 and mini-AQLQ for asthma
- CAT for COPD
- K-BILD for Interstitial lung disease
- QoL-B for bronchiectasis

7. Study procedures

7.1 Informed consent

Oral and written information is given by the responsible investigator/physician and the patient is given the opportunity to ask questions. Specifically, information as to the free will of participation, as well as withdrawal, is provided. Written informed consent is obtained before any study related measure is undertaken.

7.2 Medical history

A complete medical history is to be taken <luring the screening visit. The purpose of this is to ascertain that the subjects do not have other illnesses that will influence the results of the study.

7.3 Physical examination

A physical examination including mouth and throat, lymph nodes, lungs, heart, abdomen, skin and neurological status is done at the screening visit.

7.4 Blood samples

Venous blood samples are taken at all visits for routine clinical chemistry to exclude other conditions that mayinfluence the results of the study: Hb, TPK, WBC with diff, tryptase, ASAT, ALAT, GT, urea, creatinine, sodium, potassium, CRP. Blood samples are collected at visits 1, 2, 3, 4 and for those undergoing subsequent yearly follow-ups fora range of asthma and inflammation biomarkers, separated into plasma, serum, cells for analysis of DNA and RNA as well as immunophenotyping of lymphocyte subsets. At visit 1 a maximum of 20 mL blood will be collected. At visits 2, 3 4, and subsequent annual visits, a maximum of 90 mL blood will be collected.

7.5 Saliva samples

Collected according to Appendix 2.

7.6 Urine samples

Collected according to Appendix 3.

7.7 Exhaled breath samples

In addition to measurement of the fraction of exhaled nitric oxide (FENO), PExA, the eNose and other devises for collection of the exhaled air will be used for exploratory

studies of specific volatile and particular components of the exhaled breath. The procedures and devises are described in Appendix 4.

8. Data recording

Data consists of patient characteristics (age, gender, treatment, lung function, etc), and outcome variables (lipid mediators and other biomarkers in urine, sputum, saliva and exhaled air). Coded data are recorded in excel data sheets used as study specific CRFs. In addition, special protocols for recording of the sampling of biomarkers are used as specified in the respective appendices. It is only the responsible physicians and nurses at the research unit who will have access to the patient identification list. Data kept on paper along with the study documents will be filed in a locked cabinet in the research unit for at least ten years. Digitised data will be placed in a locked master-file after quality control and data cleaning have been concluded prior to breaking the study code. This file is stored behind password protected firewalls at the server of the university. Changes in the master-file will be documented and can only be done by a few project managers with delegation rights.