Research Project

Efficacy of Slow-Release L-cysteine (Acetium™ Capsules)* in Prevention of Exacerbation of Bronchial Asthma. A randomized controlled trial.

Executed by:

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SYNOPSIS

Background: Globally, over 300 million people are affected by bronchial asthma, with some 250.000 annual deaths. Thanks to the recent developments of more effective therapies, asthma mortality has decreased substantially. Meanwhile, however, allergic diseases have markedly increased in the past half century, and children today have the highest prevalence of asthma compared with the older generations. Unfortunately, it is anticipated that the number of asthma patients will increase by more than 100 million until 2025.

Several different forms of asthma are included in the IDC-10 classification, two main categories being allergic and non-allergic asthma. In 1995, the **Global Initiative for Asthma (GINA)** developed a Global Strategy for Asthma Management and Prevention, including a widely accepted classification of asthma by severity: i) intermittent, ii) mild persistent, iii) moderate persistent or iv) severe persistent. In their subsequently revised evidence-based guidelines, GINA concludes that clinically more relevant is an accurate classification of **asthma control**, as: i) controlled, ii) partly controlled, and iii) uncontrolled. In the latest guidelines of 2010, GINA recommends that achieving **overall asthma control** is the **goal of all therapy**, based on 5 Steps. Step 1 is as needed rapid-acting inhaled β2-agonist. The other 4 treatment steps include a controller option, ranging from low-dose inhaled corticosteroids (ICSs) as the preferred treatment option at Step 2, to high-dose ICSs plus long-acting inhaled β2-agonist combinations together with oral corticosteroids at Step 5. Once the level of asthma control has been established, consideration should be given to reducing the amount of treatment. By contrast, if asthma is uncontrolled, treatment needs to be increased to the next step.

In pathogenesis of asthma, **histamine** (and other mediators) liberated from tissue **mast cells** plays a key role in the development of the three **clinical hallmarks** of full-blown asthma; 1) airway inflammation, 2) bronchial hyper-reactivity, and 3) obstruction. **At tissue level**, these mast-cell mediators induce i) vasodilation, ii) contraction of the bronchial smooth muscle, and iii) mucous secretion by the bronchial mucous glands. At **molecular level**, histamine is a potent trigger of epithelium-derived **oxidative stress,** mediated by histamine receptors H1R and H4R. IgE-mediated cross-linking of the membrane bound IgE high-affinity receptor (FcεRI) is the best characterized pathway of mast cell activation and degranulation in asthma, but there are other potent **liberators of histamine** as well, including **acetaldehyde**. Substantial experimental and clinical data leave little doubt that acetaldehyde is a potent inducer of **bronchoconstriction** in asthmatic patients by liberating histamine from the mast cells in the airways, and this would neatly explain the mechanism whereby alcohol intake can trigger asthma exacerbation (attack).

Acetaldehyde can be effectively **eliminated** in the stomach contents after alcohol intake by using a semi-essential amino acid, L-cysteine; a principle patented by Biohit Oyj in their Acetium™ capsule (containing 100mg L-cysteine in slow-release formulation). In addition to eliminating acetaldehyde, L-cysteine is one of the most **potent antioxidants** among the thiol-compounds. Interestingly, spontaneous case testimonials from migraine patients recently testified that regular use of Acetium™ capsules proved to be highly effective against **migraine** attacks. Acute headache in migraine is another **histamine-related** reaction (triggered by an abrupt vasodilation in cerebral blood vessels), and this analogy to asthma prompted us to formulate the hypothesis for the present RCT of asthmatic patients using Acetium™ capsules.

It is tempting to speculate that reducing the total acetaldehyde burden in the body by Acetium™ capsules in the stomach (and Acetium lozenges in the saliva among smokers), might critically contribute to maintaining the histamine levels below the threshold that disturbs the sensitive equilibrium between **histamine-mediated bronchoconstriction** vs. no bronchoconstriction, i.e., exacerbation or not of asthma, respectively. Furthermore, the **antioxidant** property of L-cysteine might be important in protecting the lungs against the (histamine-induced) **oxidative injury** inherent to the chronic inflammation in airways of asthmatic patients.

Objective: The present study is designed to validate this novel hypothesis that daily use of L-cysteine (Acetium capsules, 100mg twice a day, and Acetium lozenges among smokers) is an effective means to decrease the frequency of (or completely abort) the histamine-mediated attacks of asthma.

Study design: A double-blind, randomized placebo-controlled clinical trial comparing Acetium capsules (100mg Slow-Release L-cysteine, twice a day) and placebo in control of asthma during a 3-month trial period. A cohort of 200 voluntary subjects (both genders) are invited through the Allergy and Asthma Federation (AAF), to participate in the study. To be eligible, the subjects should: i) have **partly controlled** or **uncontrolled** asthma (GINA), ii) have their asthma diagnosed for at least 12 months, iii) have the onset of their asthma before 50 years of age, iv) be between 18 and 65 years of age, and v) have a minimum of co-morbidity. Before enrolment in the cohort, all subjects are requested to sign a written consent. The study protocol will be subjected for approval by the National Committee on Medical Research Ethics (TUKIJA).

Methods: A 3-month retrospective history and 1-month prospective baseline (run-in) period is used to assess the baseline asthma control (attach frequency). The study setting is actually triple-blinded (participant-blind, investigator-blind, sponsor-blind). Placebo preparation with design and package identical to the test preparation will be used. Parallel group design instead of cross-over design is used. Randomization will be performed after the 1-month prospective run-in time, using a random number generator, with blocks size of 4, and creating unique randomization codes for each subject. To control for gender differences, a stratified randomization is used, gender as the stratification variable.

The treatment period in both study arms will be 3 months. All patients should continue their regular treatment tailored according to the level of asthma control (GINA), including their usual symptomatic or acute treatment. During the 3-month treatment period, participants will be evaluated at monthly intervals by the study coordinator. As determined by the final study compliance, data analyses will be done separately for i) Per Protocol (PePr), and ii) Intention-to-treat (ITT) groups.

In addition to the baseline assessment of asthma control (by 1-month diary, spirometry), each subject will be requested to fill in a structured Questionnaire recoding their detailed asthma history and other pertinent data on potential triggers, to be used as covariates in multivariate analysis. The asthma diary is the main research tool used to monitor the efficacy of the test preparations, recording all predefined assessment measures (efficacy, tolerability and safety). These diaries also include the daily records of **daytime asthma symptom scores** and **nocturnal awakenings,** as well as the peak expiratory flow rate (PEFR) measurement twice a day (morning and evening). In addition, asthma control is monitored at baseline, randomization, and at 1-month intervals by spirometry as well as using three internationally validated classification tools: the Asthma Control Test™ (ACT), the GINA guidelines, and the (adult) Asthma Therapy Assessment Questionnaire (ATAQ).

In statistical analysis, both conventional techniques (e.g. non-parametric paired-samples and non-paired samples t-test), and more sophisticated methods will be used. The latter include i) life-table methods like Kaplan-Meier and Cox proportional hazards regression, as well as ii) generalized linear models (GEE and panel Poisson), and as a new technique in asthma RCTs, a competing risks regression, to model the asthma control during the intervention. This study (n=100 per study arm) is adequately powered (Type II error 0.80, type I error 0.05) to detect a true difference in proportion of **exacerbation days** between 0.40 in the placebo and 0.22 in the Acetium arm, i.e., the difference in effect size of 18%. Within this effect range, the study power is sensitive to any decrease in this difference and also critically dependent on the SD in the two arms. Given that the study subjects are selected among patients with partly controlled or uncontrolled asthma, these figures seem reasonable estimates for the basis of these power calculations.

Specific aims: The null hypothesis of the study implicates that L-cysteine is no better than placebo in controlling bronchial asthma during the intervention period of 3 months. Rejection or not of the null hypothesis is based on comparison of the two arms for the primary study endpoint and (to lesser extent) for a series of secondary endpoints. The pre-specified **primary efficacy endpoint** is the **percentage of asthma exacerbation days**, defined as the days when **<u>any</u>** of the following occur: i) awake all night (awake all night or recurrent episodes of awakening)(nocturnal scale), ii) increase of >50% from baseline in the symptom score (daytime scale), iii) increase from baseline in β-agonist use of >70% (minimum increase 2 puffs/day), iv) decrease from baseline of >20% in the morning PEFR, v) morning PEFR <180 l/min, or vi) an asthma attack

(i.e., unscheduled medical care for asthma). In addition to this definition (100), ad hoc analyses will be done also using two alternative definitions of an asthma exacerbation day (102-104). The treatment efficacy is also analysed for a series of **secondary efficacy endpoints**, of which **the number of asthma-free days** is the most relevant. An asthma-free day is defined as a day when **all** of the following occur: i) no nocturnal waking, ii) use of two puffs or less of β-agonist, iii) no use of oral corticosteroids, and iv) no unscheduled use of medical care for asthma (=no asthma attack).

Study execution and time table: Meanwhile the final protocol is under evaluation for ethical approval by TUKIJA, some preparatory measures will be initiated, including the information of AAF, to encourage the asthma patients who are interested in participating to contact the study coordinator. Our aim is that the required cohort of volunteers will be available within a short period since the completion of the formalities for Ethical Committee approval, or during the first quarter of 2014. Given that each study subject shall complete the 3-month treatment period, preceded by 1-month run-in time, we expect that the study will be completed during the second half of 2014.

Impact of the study: Given that L-cysteine is a natural (semi-essential) amino acid, converted to inert substance (MTCA) in the alimentary tract, it would comprise an ideal means to conduct treatment for asthma control for years, without concern about the side effects that are inherent to some of the current treatment modalities. If the efficacy is established in this formal RCT, the concept of using Acetium capsules would represent a major step forward in a better clinical control of this frequently debilitating syndrome.

1.BACKGROUND

Asthma is characterized by the action of airway leading to reversible airflow obstruction in association with bronchial hyper-responsiveness (BHR) and airway inflammation (1). The disease is affecting more than 300 million persons all over the world, with approximately 250,000 annual deaths (2). In the PAST couple of decades, as the inhaled corticosteroid has become the major treatment modality of asthma, the mortality of asthma has decreased substantially (3). Meanwhile, however, allergic diseases, such as asthma, have markedly increased in the past half centuries associated with urbanization (4). Children have the greatest percentage of asthma compared with other generation groups (Centers for Disease Control and Prevention, 2011). Then, it is expected that the number of the patients will increase by more than 100 million by 2025, unfortunately (2,5).

The etiology of asthma is multifactorial and involves a complex interaction between predisposition genes, early-life events and environmental exposures (6,7). Status of asthma is reflected by different subjective and objective indicators, and conventionally assessed by the frequencies of night-time awakening, daytime symptoms and exercise limitation (8) as well as quality of life scores (9). However, asthma can also be assessed objectively by medication use, school or work absences, peak expiratory flow (PEF) monitoring, spirometry and acute bronchodilator response to a short-acting β2-agonist (6). Other asthma biomarkers include direct (histamine, methacholine), indirect (adenosine, hypertonic saline) and specific (inhalant allergen) challenges for the presence and extent of BHR. Routine monitoring measures of asthma include history and physical examination, spirometry and home PEF diaries (5-8). The pathogenesis of asthma involves both inflammatory and non-inflammatory components, with airway inflammation being a central feature. Treatment for asthma includes anti-inflammatory medications such as inhaled corticosteroids (ICSs). Nonetheless, monitoring of airway inflammation should therefore be part of the management plan for all asthmatic patients (2,5,8).

1.1.Classification of asthma by type of exposure

Asthma is thought to be caused by a combination of genetic and environmental factors (10). Its diagnosis is usually based on the pattern of symptoms, response to therapy over time, and spirometry (2,5,8,11). Asthma is clinically classified according to the frequency of symptoms, forced expiratory volume in one second (FEV_1), and peak expiratory flow rate (12). Asthma may also be

classified as atopic (extrinsic) or non-atopic (intrinsic)(13), where atopy refers to a predisposition toward developing type 1 hypersensitivity reactions.

While asthma can also be classified based on severity (see Section 1.2), at the moment there is no clear method for classifying different subgroups of asthma beyond this system (14). Finding ways to identify subgroups that respond well to different types of treatments is a current critical goal of asthma research (14). Although asthma is a chronic obstructive condition, it is not considered as a part of chronic obstructive pulmonary disease that are irreversible such as bronchiectasis, chronic bronchitis, and emphysema. Unlike these diseases, the airway obstruction in asthma is usually reversible; however, if left untreated, the chronic inflammation from asthma can lead the lungs to become irreversibly obstructed due to airway remodeling (2,5,8,14). Importantly, in contrast to emphysema, asthma affects the bronchi, not the alveoli.

1.1.1.Atopic asthma

Atopy refers to changes in some of the genes that make their immunological system to produce abnormal amounts of Immunoglobulin E (IgE). This inherited propensity make these people more sensitive to different exposures like chemicals, smoke and dust (environmental antigens). This hypersensitivity means they are more sensitive or allergic to things in the environment than people who do not have these changes in their genes. Usually a person who is atopic develops allergic rhinitis which affects the nasal passages and they are also more likely to get atopic dermatitis, with skin rashes. Up to 40% of the people with allergic rhinitis also have asthma (2,5,8,15,). These three medical problems, allergic rhinitis, atopic dermatitis and atopic asthma are called the Atopic Triad (16). Atopic people tend to have other medical problems including food and drug allergies, stinging-insect hypersensitivity, hives (urticaria), Quincke's edema (angioedema), and contact dermatitis. The probability of being atopic increases if one or both parents are atopic.

1.1.2.Cough-variant asthma

Cough-variant asthma is a type of asthma in which a cough is the main, and sometimes only sign. Cough-variant asthma usually does not cause wheezing or breathlessness and causes a dry, scratchy, mostly nonproductive cough (this means little or no phlegm is coughed up). About 30% of people who have cough-variant asthma will eventually develop typical asthma (17).

1.1.3.Work-related asthma

Work-related asthma (WRA) includes the cases of disease that are caused or made worse by irritants in the environment at work. The jobs that are likely to cause WRA are usually those in which there is a lot of smoke or chemicals are used. Four types of WRA are distinguished (18).

1.1.3.1.Occupational asthma with latency

This type of asthma includes cases when the signs and symptoms of asthma occur after a period of time (latency) after being exposed to the environmental irritants (19). This latency period is usually a few months but can extend to several years after initial exposure.

1.1.3.2. Irritant-Induced Asthma (IIA)

This is the occupational asthma without latency, characterized by the onset of the signs and symptoms of asthma immediately after being exposed to the environmental irritants (20).

1.1.3.3. Reactive Airways Dysfunction Syndrome (RADS)

This is a term proposed in 1985 by Brooks et al. to describe an asthma-like syndrome developing after a single exposure to high levels of irritating vapor, fume or smoke (21).

1.1.3.4. Work-aggravated asthma:

This condition applies to situations when a person already has asthma, and environmental triggers at their work make it worse.

1.1.4.Exercise-induced asthma

Exercise induced asthma (EIA) is also known as exercise-induced bronchospasm, and used to define the special cases of asthma, in which exercise is the main (and many times the only) trigger for an asthma attack. If a person already has asthma or if he/she is atopic, the chance of getting EIA in considerably increased (2,5,8).

1.1.5.Nocturnal asthma

Nocturnal asthma is the term used to describe special forms of asthma that are getting worse at night (2,5,8).

1.1.6.Premenstrual asthma (PMA)

This condition may affect up to 40% of female asthma sufferers, and denotes the cases when asthma symptoms get aggravated during the premenstrual period. For an accurate diagnosis of PMA, it is essential to have a detailed history of the timing of menstrual cycles along with asthma symptoms, and the peak expiratory flow rate (PEF). Keeping a diary of symptoms and PEF rates facilitates the correct diagnosis (22).

1.1.7.Status asthmaticus

Status asthmaticus is a severe form of asthma in which an asthma attack gets worse as it goes along and the medicines that are usually used to treat asthma do not work. Status asthmaticus can be fatal (2,5,8,14).

1.2.Classification of asthma by severity

In 1993, the Global Initiative for Asthma (GINA) was implemented to develop a network of individuals, organizations and public health officials for the dissemination of information regarding the care of patients with asthma, while at the same time assuring a mechanism to incorporate the results of scientific investigations into asthma care. In 1995, in collaboration with the National Heart, Lung, and Blood Institute of the USA and the World Health Organization (WHO), GINA developed a Global Strategy for Asthma Management and Prevention; this 1995 report was first revised in 2002 (23), and subsequently in 2006 (24). The 2002 GINA report stated: ''it is reasonable to expect that in most patients with asthma, control of the disease can and should be achieved and maintained'' (23). To meet this challenge, the 2006 report not only incorporated updated scientific information but also described a development of this theme and a change in approach to asthma management, with asthma control, rather than asthma severity, being the focus of treatment decisions (24).

In their 2008 update (7), GINA executive summary also revisited their classification of asthma by the disease severity. Previous GINA documents subdivided asthma by severity, based on the level of symptoms, airflow limitation and lung function variability, into four categories: i) intermittent, ii) mild persistent, iii) moderate persistent or iv) severe persistent (23,24). In the 2008 update, GINA admitted that this classification, was based on expert opinion rather than evidence (7). Accordingly, classification of asthma by the severity of symptoms is useful when decisions are being made about

management at the initial assessment of a patient. However, it is important to recognize that asthma severity involves both i) the severity of the underlying disease and ii) its responsiveness to treatment (7). Importantly, severity is not an unchanging feature of an individual patient's asthma but may change over months or years. Because of these considerations, the classification of asthma by severity was no longer recommended as the basis for ongoing treatment decisions. However, it may retain its value as a cross-sectional means of characterizing a group of patients with asthma who are not on inhaled glucocorticoid treatment, for selecting patients for inclusion in asthma studies. Its main limitation is its poor value in predicting what kind of treatment will be required and what a patient's response to that treatment might be (7,8,23,24).

1.3.Symptoms of asthma

Asthma is characterized by recurrent episodes of wheezing, shortness of breath, chest tightness, and coughing (2,5,7,8,23,24). Sputum may be produced from the lung by coughing but is often hard to bring up. During the recovery from an attack, it may appear pus like due to high levels of white blood cells called eosinophils (7,8). Some people with asthma may have long periods of time between asthma attacks where they show no signs and experience no symptoms of asthma, while others may have some or all of the signs and symptoms every day which become more severe during an attack. A person may have some signs and symptoms during one asthma attack and have different symptoms during another asthma attack. It also depends on what type of asthma (16-22) a person has and whether they have a mild, moderate or severe asthma (7,23,24).

There are also some people with asthma who might only have signs and symptoms during certain times, such as those with EIA, where the exercise triggers the symptoms. For some others, the signs and symptoms of asthma may be triggered or made worse (exacerbated) when they have viral respiratory tract infections, often caused by human rhinoviruses (7,8). Symptoms are usually worse at night and in the early morning or in response to exercise or cold air. Some people with asthma rarely experience symptoms, usually in response to triggers, whereas others may have marked and persistent symptoms (2,5,7,8,23,24).

1.3.1.Associated conditions

Among people with asthma, it is not unusual to have a number of other health conditions (co-morbidity)(7,8), including gastro-esophageal reflux disease (GERD), rhino-sinusitis, and obstructive

sleep apnea (25). Psychological disorders are also more common, with anxiety disorders occurring in between 16–52% and mood disorders in 14–41% (26). However, it is not known if asthma causes psychological problems or if psychological problems lead to asthma (7,8). In the above, mention was already made of different hypersensitivity syndromes associated with atopic (intrinsic) asthma.

1.3.2.Early warning signs

Early warning signs of an asthma attack are physical changes in feelings or in regular health conditions that an asthmatic person has before an attack. By being aware of these early warning signs, a person may be able to take the necessary steps to avoid having an asthma attack, or if the attack is unavoidable, to keep its severity under control.

Such early warning signs of asthma may include: 1) coughing a lot, especially at night; 2) losing one's breath easily; 3) shortness of breath; 4) getting tired easily during exercise and feeling weak and wheezing or coughing after exercise; and 5) feeling the symptoms of a cold or allergies coming on like sneezing, a runny or stuffed up nose, coughing, sore throat, and headache (2,5,7).

1.4.Causes and triggers of asthma

The exact cause of asthma is not yet known. It is believed that asthma is caused by a combination of complex and incompletely understood environmental and genetic interactions (10,27). These factors influence both its severity and its responsiveness to treatment (7,8,28). It is believed that the recently increased prevalence rates of asthma (2,5) are due to changing epigenetics (i.e., heritable factors other than those related to DNA) and changing living environments (29).

1.4.1.Genetic predisposition

Family history is a risk factor for asthma, with many different genes being implicated (30). If one identical twin is affected, the probability of the other having the disease is approximately 25%. By the end of 2005, 25 genes had been associated with asthma in six or more separate populations, including GSTM1, IL10, CTLA-4, SPINK5, LTC4S, IL4R and ADAM33, among others (31). Many of these genes are related to the immune system or involved in modulation of the inflammation. Even among this list of genes, however, the results have not been consistent among all studied populations. In 2006, over 100 genes were associated with asthma in one single genetic association study alone (31), and it is obvious that many more continue to be discovered (32).

Some genetic variants may only cause asthma when they are combined with specific environmental exposures (10). An example is a specific single nucleotide polymorphism in the CD14 region and exposure to endotoxins. Endotoxin exposure can be derived from several environmental sources including tobacco smoke, dogs, farms, etc. Thus, the risk for asthma is determined by both a person's genetics predisposition and the level of endotoxin exposure (10).

1.4.2.Environmental

Many environmental factors have been associated with the development and exacerbation of asthma, including allergens, air pollution, and other environmental chemicals (33). Smoking during pregnancy and after delivery is associated with a substantially increased risk of asthma-like symptoms (7,8). Low air quality because of traffic pollution or high ozone levels (8), has been associated both with asthma development and its increased severity (34). Similarly, exposure to indoor volatile organic compounds may be a trigger for asthma; e.g. formaldehyde exposure has a positive association (35). Also, phthalates in PVC are associated with asthma in children and adults (36) as are high levels of endotoxin exposure (37).

There is little doubt that asthma is associated with exposure to indoor allergens (38). Common indoor allergens include: dust mites, cockroaches, animal dander, and mold (39). Efforts to decrease dust mites have been found to be ineffective (40). Certain viral respiratory infections, such as respiratory syncytial virus and rhinovirus, may increase the risk of developing asthma when acquired as young children (7,8). Certain other infections, however, may decrease the risk (23,24).

1.4.2.1.Hygiene hypothesis

The hygiene hypothesis was introduced some years ago to explain the increased global rates of asthma as a direct and unintended result of reduced exposure (during childhood) to non-pathogenic bacteria and viruses (41,42). It has been proposed that the reduced exposure to bacteria and viruses is due, in part, to increased cleanliness and decreased family size in modern societies (43). Circumstantial evidence substantiating the hygiene hypothesis includes lower rates of asthma among people living in farms and in households with pets (43).

Use of antibiotics in early life has been linked to the development of asthma (44). Also, delivery via caesarean section is associated with an increased risk (estimated at 20–80%) of asthma, being

attributed to the lack of healthy bacterial colonization acquired from passage through the birth canal (45). Interestingly, there seems to be a link also between asthma and the degree of prosperity (46).

1.4.3.Medical conditions

A triad of atopic eczema, allergic rhinitis and asthma is called atopy (2,5,8,14). The strongest risk factor for developing asthma is a history of atopic disease (2,5,8,23,24), asthma occurring at a much greater rate among those who have either eczema or hay fever (7,8). Individuals with certain types of urticaria may also experience symptoms of asthma (47). There is a correlation between obesity and the risk of asthma with both having increased in recent years (48). Several factors may be involved, including decreased respiratory function due to accumulation of fat, as well as the fact that adipose tissue leads to a pro-inflammatory state (49).

Beta blocker medications such as propranolol can trigger asthma in those who are susceptible (50). However, cardio-selective beta-blockers appear safe in those with mild or moderate disease (51). Other medications that can cause problems include ASA, NSAIDs, and angiotensin-converting enzyme inhibitors (52).

1.4.4.Triggers of asthma

Some individuals will have stable asthma for weeks or months and then suddenly develop an episode of acute asthma. Different individuals react differently to various factors (53). Most individuals can develop severe exacerbation from a number of triggering agents. Common triggers for asthma are derived from several sources.

1.4.4.1.Tobacco smoke

One of the most common trigger is tobacco smoke. An asthmatic person does not need to smoke himself, but an exposure to passive smoking can trigger an asthma attack (7,8).

1.4.4.2.Pets

Domestic animals give off proteins which can be strong allergens, acting as irritants and making someone's asthma worse and trigger an asthma attack. Except in the pet's dander, these are also found in their urine, feces, saliva, and sebum, which when becoming airborne, will be breathed in

the airways. Some of the typical pets to which people can be allergic include: dogs, cats, gerbils, hamsters, guinea pigs and pet birds (53).

1.4.4.3.Bugs

Different types of bugs which may be found inside homes may trigger asthma attacks. They may trigger asthma symptoms in the same way as pets; the proteins they give off are allergens and become airborne. Some of the more common bugs which may trigger asthma are dust mites, cockroaches and also bedbugs and fleas. Many other species that may infest a home may serve as a source of allergens, like the recently identified Pharoah ants (54).

1.4.4.4.Fungus spores (mold)

Fungi reproduce by releasing spores into the air, and breathing in these spores can trigger asthma. One of the most common types of fungus spores found in both outside and outside environments are from a group (genus) known as aspergillus (53).

1.4.4.5.Strong emotions

Sometimes, no other trigger can be identified, but strong emotions like anger, stress and even laughter may worsen the symptoms of asthma (2,5).

1.4.4.6.Outdoor air pollution

In some patients, outdoor air pollution can be a trigger for asthma attack. This pollution can be derived from many sources such as car and truck fumes, chemicals in the air near factories and refineries, etc.

1.4.4.7.Weather

In some sensitive individuals, even the changes in the weather can trigger an asthma attack. This does not need to be cold air, but even changes in air temperature can trigger an attack. Sudden changes in humidity also plays a part (55).

1.4.4.8.Alcohol

The first data suggesting that alcohol intake can induce asthma were derived from Japan, where a large proportion of inhabitants are bearers of ALDH2 deficiency, making them incapable of

metabolizing acetaldehyde to acetic acid. Accordingly, it was reported that acetaldehyde may be a main factor of alcohol-induced bronchoconstriction in Japanese patients with asthma (56,57). In elegant clinical experiments, it was concluded that acetaldehyde causes bronchoconstriction indirectly via **histamine release** in asthmatics, and that nonspecific bronchial hyper-responsiveness is a necessary precondition for the expression of acetaldehyde-produced bronchoconstriction (56). Using oral ethanol challenge test, another group concluded that alcohol-induced bronchial asthma seems to develop through alcohol-induced elevated blood acetaldehyde levels, which leads to degranulation of mast cells (or basophils), with liberation of chemical mediators such as **histamine** (57). This key role of histamine in alcohol-induced asthma was subsequently confirmed by blocking the H1-receptors by an anti-histamine (azelastine hydrochloride)(58). This resulted in dramatic reversal of the symptoms, implicating that anti-histamine agents seem to be effective against alcohol-induced asthma by both blocking H1 receptors and inhibiting the histamine release.

It is now commonly agreed that alcoholic drinks are capable of triggering a wide range of allergic and allergic-like responses, including rhinitis, itching, facial swelling, headache, cough and asthma not only in Asiatic people but in Caucasians as well. (59-61). In surveys among asthmatics, over 40% reported the triggering of allergic or allergic-like symptoms following alcoholic drink consumption and 30-35% reported worsening of their asthma (59). In Caucasians, specific non-alcohol components are the main cause of sensitivities to alcoholic drinks. Wine is clearly the most commonly reported trigger for adverse responses, particularly **histamine** in wine has been associated with the triggering of a wide spectrum of adverse symptoms, including sneezing, rhinitis, itching, flushing, headache and asthma. However, the etiology of wine-induced asthmatic responses may be complex and may involve several co-factors (59-61).

1.5.Exacerbation of asthma (asthma attack)

An acute asthma exacerbation is commonly referred to as an asthma attack. The classic symptoms are shortness of breath, wheezing, and chest tightness (7,8,23,24). While these are the primary symptoms of asthma, some people present primarily with coughing, and in severe cases, air motion may be significantly impaired such that no wheezing is heard (62).

Signs which occur during an asthma attack include the use of accessory muscles of respiration (sternocleidomastoid and scalene muscles of the neck), there may be a paradoxical pulse (a pulse

that is weaker during inhalation and stronger during exhalation), and over-inflation of the chest (63). A blue color of the skin and nails may occur from lack of oxygen.

In a mild exacerbation, the peak expiratory flow rate (PEFR) is ≥200 L/min or ≥50% of the predicted best. Moderate is defined as between 80 and 200 L/min or 25% and 50% of the predicted best, while severe is defined as ≤ 80 L/min or ≤25% of the predicted best (63).

Acute severe asthma, previously known as status asthmaticus, is an acute exacerbation of asthma that does not respond to standard treatments of bronchodilators and corticosteroids (64). Half of cases are due to infections with others caused by allergen, air pollution, or insufficient or inappropriate medication use.

1.6.Pathogenesis of asthma

Histopathological changes in the bronchi of asthmatic patients are typical and well characterized (65). The disease is considered as an inflammatory disease in the airway, leading to airway hyper-responsiveness, obstruction, mucus hyper-production and airway wall remodeling. The presence of airway inflammation in asthmatic patients has been found in the nineteenth century. As the information in patients with asthma increase, paradigm change in immunology and molecular biology have resulted in an extensive evaluation of inflammatory cells and mediators involved in the pathophysiology of asthma. Moreover, it is recognized that airway remodeling into detail, characterized by thickening of the airway wall, can have profound consequences on the mechanics of airway narrowing and contribute to the chronic progression of the disease. Epithelial to mesenchymal transition plays an important role in this airway remodeling (65). These epithelial and mesenchymal cells cause persistence of the inflammatory infiltration and induce histological changes in the airway wall, increasing thickness of the basement membrane, collagen deposition and smooth muscle hypertrophy and hyperplasia. Resulting of airway inflammation, airway remodeling leads to the airway wall thickening and induces increased airway smooth muscle mass, which generate asthmatic symptoms. Asthma is classically recognized as the typical Th2 disease, with increased IgE levels and eosinophilic inflammation in the airway. Emerging Th2 cytokines modulate the airway inflammation, which induces airway remodeling. Biological agents, which have specific molecular targets for these Th2 cytokines, are available and clinical trials for asthma are ongoing. However, the relatively simple paradigm has been doubted because of the realization that

strategies designed to suppress Th2 function are not effective enough for all patients in the clinical trials. In the future, it is required to understand more details for phenotypes of asthma.

1.6.1.Mast cells in asthma

With the changing paradigm in the pathogenesis of asthma, significant new data have been elaborated also on the complex functions of mast cells, classically considered as liberators of histamine upon stimulation by antigen-bound IgE antibody molecules (65). Mast cells play a fundamental role in the occurrence of allergic diseases because of their hypersensitive response to otherwise innocuous substances that induces an allergic reaction.

The allergic reaction begins with the interaction of allergen with polyvalent IgE–FceRI complexes expressed on the surface of sensitized mast cells that causes receptor aggregation (66). A complex signaling cascade follows involving the activation of numerous signaling proteins such as spleen TK (Syk) and Lyn kinase, which in turn cause a series of downstream signal transduction events within the mast cell. Ultimately, this signal transduction process leads to calcium influx and release of preformed chemical mediators such as **histamine** from mast cells as well as the synthesis of lipid mediators such as PGs and LTs and the production of cytokines and chemokines. The actions of these mediators on their receptors and surrounding tissue as well as their recruitment of other immune cells are responsible for the early and late effects of an IgE-mediated allergic reaction. Mast cells are therefore central players in both the development and maintenance of allergic diseases and are subsequently considered an attractive therapeutic target in the treatment of allergic diseases such as **asthma,** allergic rhinitis and allergic conjunctivitis.

Mast cells originate from pluripotent hematopoietic stem cells, which circulate as CD34+ precursors until they migrate into tissues where they mature to long living effector cells (66,67). Mature mast cells are classified into two subtypes depending on their location; connective tissue mast cells (CTMCs), which reside in tissues such as the skin, small bowel submucosa and peritoneal cavity, or mucosal mast cells (MMCs), which mature in mucosal tissues such as the intestinal lamina propria and **in the airways** (66). In humans, mast cell subtypes are also named according to their protease content: MCT mast cells store tryptase in their granula in contrast to MCCT cells which express both chymase and tryptase (67). MCCT are found in the skin, lymph nodes and submucosa of stomach and intestine. In contrast, **MCT** appear predominantly **in the lung** and the intestinal

mucosa in close proximity to other immune cells such as T cells. These subtypes vary in their sensitivity of activation and mediator profile (66,67).

1.6.1.1.Mediators in mast cells

Mast cells interact with their environment by a host of mediators. Some of them are stored in mast cell granula and some are produced de novo following activation. Mast cell mediators can be divided into the following classes; a) preformed substances, b) lipid mediators, and c) cytokines and chemokines. Like heparin, chymase, tryptase and carboxipeptidase-A, also **histamine** (and other amines) is stored in preformed form in the granula and can be released within minutes following mast cell activation.

Depending on the type and strength of stimulation, mast cells are able to release different mediator patterns within minutes. Mast cell activation and degranulation following IgE-mediated crosslinking of the membrane bound IgE high affinity receptor (FcεRI) is the best characterized pathway of mast cell activation, typically seen in asthma (66,67). Cross-linking can be mediated by bi- or multivalent antigens, recognized by membrane-bound IgE molecules or nonspecifically through super antigens. Following activation, granules fuse with the cell membrane and release their stored mediators within minutes.

1.7.Histamine and asthma

In human airways, **mast cells** can be found adjacent to blood vessels in the lamina propria of airway mucosa. Interestingly, in patients with asthma, mast cells also migrate into other structures like airway epithelium, the mucous glands and airway smooth muscle (67). This anatomical proximity to these key structures involved in asthma and direct interaction between mast cells and airway smooth muscle cells suggest that mast cells play a significant role in the pathophysiology of this disease. Mast cells and smooth muscle cells interact in a crosstalk as mast cells can induce TGF-β1 expression in smooth muscle cells via release of β tryptase, resulting in differentiation of the muscle cells into a more contractile phenotype. Moreover, airway smooth muscle cells can enhance mast cell survival in a cell contact dependent manner and can induce mast cell degranulation, representing a new antigen-independent type of mast cell activation.

In patients with allergic asthma, inhalation of an aeroallergen leads to cross-linking of membrane

bound IgE via the allergen, inducing rapid release of mast cell mediators such as **histamine**, leukotrienes, proteases and prostagladins, which can be detected in increased concentration in the broncho-alveloar lavage (BAL) of allergen-challenged patients. These mediators induce **vasodilation, contraction of the smooth muscle, and mucous secretion**. Moreover, these mediators also lead to the late phase response which is characterized by infiltrating inflammatory cells, eosinophils, CD4+ T cells, neutrophils, mast cells and basophils which are associated with **swelling of the bronchial wall** and increased non-specific **airway hyper-responsiveness** (AHR)(65-67).

The key mast cell-produced mediator, **histamine**, is known to act on different cell types via **four distinct histamine receptors** (**H1R-H4R**)(66,67). Depending on the expression level of the receptors and the cell type, histamine can have different effects with pro- but also anti-inflammatory patterns. In regard to DC (dendritic cell) activation, H1R and H3R induce pro-inflammatory responses with increased antigen presentation, cytokine production and Th1 priming activity, whereas activation of H2R induces IL-10 secretion and a regulatory DC phenotype (65-67). In T cells, depending on the receptor expression pattern, histamine can induce the production of Th1 cytokines such as IFN-γ or Th2-specific cytokines like IL-4 and IL-13. Recently, it has also been demonstrated that pro-inflammatory effects of mast cell-derived histamine might be mediated by suppressing CD4+ CD25+ regulatory T cells. In allergic airway disease, Bryce, et al. (2006) demonstrated an important role of histamine acting via **H1 receptor** (68). Indeed, H1-receptor-deficient animals were not able to allergic airway disease following sensitization and challenge. Especially, H1 receptor-deficient animals showed a defect in T cell migration into the lung. Furthermore, **H4 receptor** also seems to play an important role in the histamine-dependent induction of allergic airway disease. Consequently, novel H4 receptor antagonists have been developed and have been shown to be effective in suppressing the development of allergic airway disease in murine models (69,70).

1.8.Liberators of histamine: acetaldehyde

Most histamine in human body is generated in granules of the **mast cells** (in tissues) or in white blood cells called basophils (66,67). Mast cells are especially numerous at sites of potential injury, including the nose (and other airways), mouth, **gastrointestinal tract**, skin, and **blood vessels**. Non-mast cell histamine is found in several tissues, including the brain, where it functions as a

neurotransmitter. Another important site of histamine storage and release is the **enterochromaffin-like (ECL)** cells of the gastric corpus. These cells bear a surface receptor for gastrin, whereby gastrin-17 stimulates liberation of their histamine content, which in turn, stimulates acid secretion by the parietal cells in the corpus mucosa.

As discussed above, mast cells play a crucial role in many **hypersensitivity**, allergic, and inflammatory reactions by secreting chemical mediators, e.g. histamine as a response to various immunologic and non-immunologic stimuli. Given the fact that also alcohol intake induces (especially in Oriental individuals with hereditarily deficient ALDH2 enzyme), **hypersensitivity** symptoms that can be blocked by antihistamines, as well as urticaria and anaphylactoid reactions in Caucasian individuals as well, led Koivisto et al. (1996) to speculate the possibility that **acetaldehyde** (the first metabolite of alcohol) might enhance these reactions by directly affecting the tissue **mast cell** functions (71). Indeed, these authors were the first to demonstrate that this is the case. Using purified rat peritoneal mast cells incubated with different concentrations of acetaldehyde and ethanol at body temperatures, they demonstrated that acetaldehyde, at relatively low concentrations (50μM), directly induces histamine release from the mast cells. Ethanol did the same, but only at molar concentrations (71).

The authors reasoned that such acetaldehyde-induced histamine release from the mast cells may contribute to different hypersensitivity reactions caused by alcohol intake, including some alcohol-associated gastrointestinal disorders (71). Subsequently, the validity of this concept has been confirmed also in other clinical conditions, including human bronchial mast cells (72). In their elegant experiments, Kawano et al. (2004) demonstrated that acetaldehyde ($>3 \times 10^{-4}$ M) increased airway muscle tone, which was associated with a significant increase in the release of histamine, but not thromboxane B2 or cysteinyl-leukotrienes. A histamine (H1-receptor) antagonist completely inhibited acetaldehyde-induced bronchial smooth muscle contraction. Acetaldehyde also induced a significant histamine release from human **lung mast cells** and their degranulation. These results strongly implicate that acetaldehyde stimulates human airway mast cells to release histamine, which is involved in bronchial smooth muscle contraction e.g. following alcohol consumption (72).

Subsequently, these important observations have been confirmed by several groups in both animal experiments (73) and in humans (74,75,76). When human bronchi and mast cells were stimulated

with acetaldehyde in vitro, acetaldehyde stimulation induced bronchoconstriction and degranulation of human mast cells (73). The authors concluded that **acetaldehyde** has potential effects on human airway by two distinct mechanisms; i) as a metabolite of alcohol, its elevation following alcohol consumption induces airway mast cells to release histamine, which results in exacerbation of asthma in susceptible populations; and ii) as a constituent of e.g. cigarette smoke, inhalation of acetaldehyde potentially increases airway inflammation. Linneberg et al. (2010) studied the genetic determinants of self-reported alcohol-induced hypersensitivity reactions by assessing the single nucleotide polymorphism (SNP) of the acetaldehyde dehydrogenase 2 (ALDH2) 487lys in population of Copenhagen (75). Their data implicated that alcohol sensitivity in Caucasians is genetically determined and suggest that a histamine-releasing effect of acetaldehyde represents a plausible biological mechanism. Inhaled acetaldehyde and adenosine 5'-monophosphate (AMP) are known to cause bronchoconstriction in asthmatics by a mechanism believed to involve histamine release from the mast cells in airways (76). In this respect, inhaled acetaldehyde seems to be less potent than AMP in causing bronchoconstriction in asthma, but the response to inhaled acetaldehyde was repeatable. The results also implicate that acetaldehyde responsiveness and AMP responsiveness are not mediated by the same pathways (76).

These experimental and clinical data leave little doubt that acetaldehyde is a potent inducer of bronchoconstriction in asthmatic patients by liberating histamine from the local mast cells in the airways, which in turn incudes the smooth muscle contraction, like in a classical asthma attack (72-76).

1.8.1.Acetaldehyde: Group 1 carcinogen (IARC)

Tobacco smoke contains several classes of carcinogens that include among others polycyclic aromatic hydrocarbons, aromatic amines, and nitrosamines. Tobacco smoke contains also high concentrations of toxic aldehydes (77). The most abundant aldehyde in tobacco smoke is acetaldehyde, and its concentration in tobacco smoke is >1,000 times greater than those of polycyclic aromatic hydrocarbons and tobacco-specific nitrosamines (78). Acetaldehyde is also the first metabolite of ethanol oxidation. It binds to DNA, forming stable DNA adducts that are observed in alcohol consumers. Numerous epidemiological studies in alcohol drinkers with alcohol dehydrogenase (ALDH2) deficiency or low alcohol dehydrogenase (ADH1B) activity provide the most compelling evidence for the carcinogenicity of acetaldehyde (75,79). This deficiency results in

the accumulation of acetaldehyde locally into the saliva during ethanol metabolism and also in markedly increased risk for many upper gastrointestinal tract cancers.

Similarly, it was recently shown that acetaldehyde from the tobacco smoke is easily dissolved into the saliva during smoking (80). Thus, toxic aldehydes could mediate the carcinogenic effect of tobacco smoke through saliva to oral cavity and from there further on to the larynx, esophagus, and stomach. Based on firm epidemiological and toxicological documentation, IARC proclaimed (in 2009) acetaldehyde as Group I carcinogen, equivalent to asbestos, formaldehyde and others (81).

1.8.2.L-cysteine eliminates acetaldehyde in the stomach and in saliva

Cysteine is a non-essential amino acid, which was shown (almost 40 years ago) to be capable of eliminating the toxicity of acetaldehyde by reacting covalently with it to form a stable 2-methylthiazolidine-4-carboxylic acid (MTCA)(82). MTCA is an inert and non-toxic compound that is eliminated from the body through feces and urine, without being absorbed into the blood circulation. This simple principle was used in the recent innovation of **Biohit HealthCare's Acetium™ capsule,** which contains 100mg L-cysteine.

In the proof-of-concept study, oral administration of Acetium was confirmed to effectively bind acetaldehyde originated from ethanol metabolism in achlorhydric stomach (83). In that setting, the mean acetaldehyde level of gastric juice was 2.6 times higher with placebo than with l-cysteine (13 vs. 4.7 μM, $p<0.05$), implicating that L-cysteine can be used to decrease acetaldehyde concentration in achlorhydric stomach during alcohol exposure.

This led the authors to examine the concept, whether it would be possible to eliminate alcohol-derived acetaldehyde also from the saliva, using L-cysteine slowly released from a special buccal (Acetium) tablet (84). Indeed, this was shown to be the case in tested volunteers, in whom, up to two-thirds of acetaldehyde (after alcohol intake) could be removed from the saliva with a slow-releasing buccal L-cysteine formulation. This should have important implications e.g. in prevention of upper GI-tract cancers among individuals with high acetaldehyde exposure (heavy drinkers, smokers)(84).

As the logical next step, Biohit Oyj developed an Acetium sucking tablet (lozenge) that releases L-cysteine into the oral cavity during smoking, and tested this formulation as a potential chemo-preventive agent against toxicity of tobacco smoke, i.e. in harm reduction (85). Seven volunteers smoked five cigarettes, and during every smoking period, sucked a blinded tablet containing 0, 1.25, 2.5, 5, or 10 mg of L-cysteine, followed by acetaldehyde analysis of the saliva at 0, 5, and 10 minutes from the beginning of the smoking. L-cysteine reduced highly significantly the salivary acetaldehyde. In fact, carcinogenic acetaldehyde could be totally inactivated in the saliva during smoking by the sucking tablet containing 5 mg of L-cysteine (85).

1.9.Management of asthma

The **Global Initiative for Asthma (GINA)** is an initiative started in the early 1990's to develop guidelines for asthma diagnosis and management that were applicable to both developed and developing countries (2,5). The first asthma guidelines were published in the mid 1980's when asthma became a recognized public health problem in many countries. The GINA was launched in 1995 as a collaborative effort between the NHLBI and the World Health Organization (WHO). A comprehensive workshop report entitled ''A Global Strategy for Asthma Management and Prevention'', first published in 1995, has been widely adopted, translated and reproduced, and currently forms the basis for many national guidelines (86). This first edition was opinion-based but the subsequent updates have been evidence-based (2,5,7,8,23,24). These new updates of the GINA guidelines are based on the control of the disease.

In the latest guidelines of 2010 (87), GINA now recommends that achieving **overall asthma control** is the goal of therapy. Overall asthma control consists of 2 domains: 1) to achieve day-to-day (or current) asthma control, and 2) to minimize the future risk measured by the absence of asthma exacerbations, the prevention of accelerated decline in lung function over time, and no side effects from medications.

1.9.1.Step 1 treatment

The GINA asthma treatment paradigm consists of **5 steps of treatment** (87). At each step a preferred option and other alternatives are identified. **Step 1** is as needed (prn) **rapid-acting inhaled β2-agonist** (RABA).

1.9.2.Step 2 treatment

The most effective controller therapy for asthma is ICS (**inhaled corticosteroids**). Low doses of ICS alone can often provide good asthma control in both children and adults, and this approach remains the treatment recommendation for GINA **Step 2.** There is no convincing evidence that regular use of combination therapy with ICS and **long-acting inhaled β2-agonists** (LABAs) provides any additional benefit for milder patients. ICS treatment not only improves current control, but greatly reduces the risk of severe asthma exacerbations (87). Another issue which needs to be considered when making a decision to start ICS treatment in mild asthma is the potential for side effects. ICSs are not metabolized in the lungs and every molecule of ICS that is administered into the lungs is absorbed into the systemic circulation.

Leukotriene receptor antagonists (LTRAs) are another treatment in Step 2, but they are less effective than low-dose ICSs. There are considerable inter-individual and intra-individual differences in responses to any therapy. While on average, ICSs improve almost all asthma outcomes more than LTRAs, there may be some patients who show a greater response to LTRAs. Currently, it is not possible to accurately identify these responders based on their clinical, physiological, or pharmacogenomics characteristics.

1.9.3.Step 3 treatment

Step 3 treatment is for those patients whose asthma is not well controlled on low doses of ICS alone. Combination therapy with ICS and a LABA is the preferred treatment option for these patients. This is because the use of combination treatment of ICS and LABA for moderate persistent asthma has also been demonstrated to improve all indicators of asthma control, when compared with ICS alone, particularly in adult asthmatics (87). Another recently described treatment approach for the management of patients at Step 3 or higher is the use of an **inhaler** containing the combination of budesonide (ICS) and formoterol (LABA), both as maintenance and as relief therapy.

There has been a lot of concern raised about the safety of LABA use in asthmatic patients. These unwanted effects have included severe asthma exacerbations requiring hospitalization, life-threatening exacerbations requiring intubations, and asthma-related death. Two meta-analyses of the effect of LABAs in combination with ICS have subsequently been reported, which did not show an increased risk for hospitalizations or serious adverse events, while the relative effect on asthma-

related mortality and asthma-related intubation and ventilation could not be assessed because of the very low frequency of these events (87).

1.9.4.Step 4 treatment

Step 4 treatment is recommended for patients not controlled on low doses of ICS/LABA combinations. The most effective approach is to increase the dose of the ICS/LABA combination. Additional add-on therapy also includes LTRAs, although the combination of ICS/LABA and LTRAs has not been extensively evaluated. A recently published study has demonstrated that a long-acting inhaled anticholinergic (tiotropium bromide) is as effective a bronchodilator as the LABA salmeterol when added to ICS (87).

1.9.5.Step 5 treatment

A small percentage of patients exists who do not respond adequately to even high doses of ICS/LABA combinations, and these patients need **Step 5** treatment. This is the population that disproportionately consume health care resources related to asthma. Often these patients will require oral corticosteroids in addition to ICS/LABA combinations, in an effort to achieve asthma control. Another potential treatment option for these patients is omalizumab, which is a recombinant humanized monoclonal antibody against IgE (88). This anti-IgE antibody forms complexes with free IgE, thus blocking the interaction between IgE and effector cells, and reduces serum concentrations of free IgE. When compared with placebo, omalizumab reduces asthma exacerbations and enables a significant reduction in the dose of ICS (87).

1.9.6.GINA guidelines (summary)

A very important component of the GINA guidelines is the recommendation that, once asthma control has been achieved, the treatment is stepped down to identify the best treatment options and doses for each patient (87). There are many fewer studies that have provided insights into the best way to step down treatment than are available for stepping up treatment. The available evidence, however, suggests that when asthma is controlled with low-dose ICS, once-daily dosing is recommended. When medium-to-high dose ICS is being used, a 50% reduction in the dose should be attempted at 3-month intervals. When a combination ICS/LABA is being used, the dose of ICS should be reduced by 50%, while maintaining the dose of the LABA. If low-dose ICS/LABA is still maintaining asthma control, the LABA can be discontinued (87).

2.STUDY HYPOTHESIS

The striking novel hypothesis of this study is simply the following: L-cysteine administered by daily intake of **Acetium™ capsules** is an effective means to prevent the attacks of asthma or improve the disease control (2,5,7,8) by significantly decreasing the attack frequency.

As described above, Acetium capsules and lozenges were originally developed for inactivation of acetaldehyde in the stomach and in the saliva after alcohol intake and smoking, respectively. This novel concept on the potential prophylactic effect of L-cysteine against asthma (based on acetaldehyde inactivation and the consequent block of acetaldehyde-induced histamine liberation from tissue mast cells), emerged purely by chance. However, the trigger to formulating this hypothesis is based on **analogous mechanisms** suspected to be operating in vascular headaches (migraine and cluster), where **histamine** is considered to play a key role as a trigger of headache attacks. Indeed, the first evidence implicating that L-cysteine might be an effective blocker of this acetaldehyde-mediated sequence of events is derived **from the patients** suffering from severe migraines (and/or intractable cluster headache), who described to us that daily intake of Acetium™ capsules (for their original indications) have completely eliminated their headache attacks (89).

These spontaneous testimonials (89) given in writing by the four persons who all have suffered from either severe migraines (3 subjects) or intractable cluster headache (Case #4) for years, prompted us to consider, whether a plausible mechanism could be discovered to explain these dramatic effects experienced by all these subjects soon after onset of Acetium intake on daily basis. Given that L-cysteine is a natural (semi-essential) amino acid, converted to inert substance (MTCA) in the alimentary tract, it would comprise an ideal means to conduct asthma management for years, without concern about the side effects that are inherent to many of the current treatment modalities (86,87). If proved in a formal randomized controlled trial (RCT), the concept of using Acetium capsules in maintenance therapy of asthma, would represent a major step forward in a better clinical control of this increasingly common syndrome (2,5,7,8,86,87).

2.1.Components of the study hypothesis

The current study hypothesis is built up of several elements, all based on solid experimental and/or clinical evidence. These elements include both i) the known pathways involved in the provocation of a characteristic attack of asthma, as well as ii) established and postulated mechanisms, how L-

cysteine administration could interfere with this sequence of events.

Irrespective of the phenotype, typical asthma is characterized by three major hallmarks; airway inflammation, bronchial hyper-reactivity and obstruction. From up-stream to down-stream, the sequence of events leading to characteristic asthma attack is as follows.

2.1.1.Histamine is a key mediator in pathophysiology of asthma

In patients with allergic asthma, exposure (inhalation) to an aeroallergen leads to cross-linking of membrane-bound IgE via the allergen, inducing rapid release from mast cells of histamine and other mediators (leukotrienes, proteases and prostaglandins), all of which can be rapidly detected in increasing concentration in the broncho-alveloar lavage (BAL) of these patients. Acting together, these mast-cell mediators induce i) vasodilation, ii) contraction of the bronchial smooth muscle, and iii) mucous secretion by the bronchial mucous glands. These same mediators also lead to the late phase response which is characterized by infiltrating inflammatory cells: eosinophils, CD4+ T cells, neutrophils, mast cells and basophils, leading to swelling of the bronchial wall and increased non-specific airway hyper-responsiveness (AHR)(65-67).

2.1.2.Histamine stimulates H_2O_2 production of bronchial epithelial cells

Besides granulocytes, also bronchial epithelium can produce large amounts of reactive oxygen species and thus contribute to asthma-related **oxidative stress**, recently implicated in pathogenesis of asthma. Recent data implicate that **histamine** could be a trigger of such an epithelium-derived oxidative stress in asthma. In their elegant experiments, Rada et al. (2013) found that air-liquid interface cultures of primary human bronchial epithelial cells and an immortalized bronchial epithelial cell line (Cdk4/hTERT HBEC) produced hydrogen peroxide in response to histamine (90). The main source of airway epithelial hydrogen peroxide is an NADPH oxidase, Duox1. Out of the four histamine receptors, **H1R** has the **highest expression in bronchial epithelial cells** and mediates the hydrogen peroxide-producing effects of histamine. **Interleukin-4** (one of the key cytokines liberated from the mast cells)(66,67) induces Duox1 gene and protein expression levels and enhances histamine-induced hydrogen peroxide production by epithelial cells (90). Using HEK-293 cells expressing Duox1 or Duox2 and endogenous H1R, histamine triggers an immediate intracellular calcium signal and hydrogen peroxide release. Over-expression of H1R further increases the oxidative output of Duox-expressing HEK-293 cells. These results indicate that

bronchial epithelial cells respond to histamine with Duox-mediated hydrogen peroxide production, providing a mechanism that could contribute to the oxidative stress characteristic of asthmatic airways (90).

2.1.3.Histamine is synthesized in tissue mast cells and basophils

Histamine is synthesized in tissue mast cells and basophils by histidine decarboxylase converting histidine to histamine. Another important source of histamine are **enterochromaffin-like (ECL) cell**s that are abundant in **gastric (corpus) mucosa**. In human airways, mast cells can be found adjacent to blood vessels in the lamina propria of airway mucosa. In patients with asthma, mast cells are also detected in close proximity to the other structures, known to be affected in pathogenesis of asthma; bronchial epithelium, mucous glands and bronchial smooth muscles (67). This anatomical proximity to these key structures involved in asthma and direct interaction between mast cells and airway smooth muscle cells suggest that mast cells play a significant role in the pathophysiology of this disease.

2.1.4.Histamine is liberated from mast cells by acetaldehyde

Most histamine in human body is generated in granules of the mast cells (in tissues) or in white blood cells called basophils (66,67). Mast cells are especially numerous at sites of potential injury, including the nose (and other airways), mouth, gastrointestinal tract, skin, and blood vessels. Histamine is liberated from the mast cells by degranulation, as a response to various immunologic and non-immunologic stimuli, asthma being a typical example.

Koivisto et al. (1996) were the first to demonstrate that acetaldehyde, at relatively low concentrations (50μM), directly induces histamine release from the mast cells. Ethanol did the same, but only at molar concentrations (71). Using bronchial mast cells, Kawano et al. (2004) demonstrated that acetaldehyde (>3 x 10–4 M) increased airway muscle tone, which was associated with a significant increase in the release of histamine (72). A histamine (H1-receptor) antagonist completely inhibited acetaldehyde-induced bronchial smooth muscle contraction, implicating that acetaldehyde stimulates human airway mast cells to release histamine, which is involved in bronchial smooth muscle contraction e.g. following alcohol consumption (72). Subsequent experimental and clinical data confirm that **acetaldehyde** is a potent inducer of

bronchoconstriction in **asthmatic** patients by liberating histamine from the local mast cells (72-76).

2.1.5.Acetaldehyde in the saliva and stomach is inactivated by L-cysteine

The phenomenon that L-cysteine eliminates free acetaldehyde by reacting covalently with it to form a stable 2-methylthiazolidine-4-carboxylic acid (MTCA)(82) was exploited by Biohit HealthCare while developing their **Acetium™ capsule** containing 100mg L-cysteine. Both the capsule and the recently introduced Acetium lozenge (5 mg L-cysteine) effectively eliminated acetaldehyde derived from alcohol and/or cigarette smoking, both in the stomach and in saliva (83-85).

2.1.6.L-cysteine has antioxidant properties

Another interesting link between L-cysteine and asthma is derived from the molecular mechanisms related to the **oxidative injury** associated with the chronic inflammation; a key characteristics of asthma (airway inflammation, bronchial hyper-reactivity and obstruction)(65-67). It has been known for some time that **thiol-containing** compounds play an important role in protecting biologic systems against oxidative injury (91,92). L-cysteine is a semi-essential amino acid, which can be biosynthesized in humans. The thiol side chain in cysteine is susceptible to oxidization, to give the disulfide derivative cysteine. Due to the ability of thiols to undergo redox reactions, cysteine has **antioxidant** properties. Oxidants and antioxidants are proposed to participate in this redox regulation by shifting the balance between reduced and oxidized cellular thiols (93). These antioxidant properties of cysteine are typically expressed in the tripeptide glutathione (GSH), reduced GSH being the most abundant intracellular low molecular weight thiol, but other thiols can also protect against oxidative injury or inhibit signal transduction.

In their elegant experiments, Winterbourn et al. (1999) examined the reactivities of several thiol-compounds (glutathione, **cysteine,** cysteamine, penicillamine, N-acetylcysteine, dithiothreitol and captopril) with superoxide generated from xanthine oxidase and hypoxanthine, and with reagent hydrogen peroxide (93). The relative reactivities of the different thiols with both oxidants were inversely related to the pK of the thiol group, penicillamine being the most reactive, while N-acetylcysteine was only weakly reactive. The reactivity of cysteine was among the top, second only to that of penicillamine (93). Taken together, it seems that in addition of being effective in inactivating acetaldehyde (=liberator of histamine, the latter increasing oxidative stress)(90), L-

cysteine seems to be one of the most **potent antioxidants** among the thiol-compounds. The latter property might be important in protecting the lungs against the (histamine-induced) oxidative injury inherent to the chronic inflammation in airways of asthmatic patients.

2.1.7.The efficacy of L-cysteine in management of asthma

We are convinced that the dramatic disappearance of migraine attacks and cluster headaches immediately after regular intake of Acetium capsules as described in the case histories (89) cannot be by change. Instead, we believe that the mechanism must be based on the capacity of L-cysteine to interfere with the attack trigger sequence by i) elimination of acetaldehyde in the stomach (or its reduction below the threshold levels), ii) blocking histamine liberation from the tissue mast cells and ECL cells in the stomach, thus arresting its multitude of functions, of which vasodilatation is critically involved in inducing the migraine attack.

Using the same analogy, it is tempting to speculate that reducing the total acetaldehyde burden in the body by Acetium capsules in the stomach (and Acetium lozenges in the saliva among smokers), might critically contribute to maintaining the histamine levels below the threshold that disturbs the sensitive equilibrium between histamine-mediated bronchoconstriction vs. no bronchoconstriction, i.e., exacerbation or not of asthma, respectively.

The present study is designed to validate this novel hypothesis that daily use of L-cysteine (Acetium capsules, 100mg twice a day, and Acetium lozenges among smokers) is an effective means to decrease the frequency of (or completely abort) the histamine-mediated attacks of asthma, analogous to those of migraine, of which anecdotal case testimonials are available, reporting complete remissions after Acetium intake (89).

3.STUDY DESIGN

This double-blind, placebo-controlled randomized trial (RCT) is designed to test the efficacy of intervention by Acetium capsules (used twice a day) in reducing the frequency of (or completely aborting) exacerbation of asthma (asthma attacks) among patients with clinically diagnosed bronchial asthma. A cohort of 200 subjects with clinically diagnosed bronchial asthma will be enrolled utilising the membership registry of the Allergy and Asthma Federation (Allergia- ja Astmaliitto ry)(AAF) and/or by public invitation, and randomly allocated (after 1-month baseline

period) to two study arms (n=100 in each), receiving either Acetium capsules or placebo. All subjects will be requested to fill in a structured questionnaire recoding their detailed asthma history and other clinical data pertinent to this study. The subjects will be administered an asthma diary on daily basis, submitted to the study monitor monthly for recording the compliance of each subject with the medication. In addition, the level of asthma control (94) will be recorded by using three validated classification tools: the **Asthma Control Test™** (ACT)(95), the **GINA guidelines** (96) and the **Asthma Therapy Assessment Questionnaire** (ATAQ)(97). The trial period will be 3 months in both study arms.

3.1.Patient selection (=criteria of being eligible)

This intervention trial is designed and conducted in conformity with the current guidelines of the GINA, by Biohit HealthCare (Helsinki, Finland), supervised by the members of the Company's Scientific Board and a consulting pulmonologist, expert in the field of asthma. These guidelines give useful recommendations for patient selection, trial design, as well as evaluation of the asthma control (98).

3.1.1.Definition of asthma

The subjects into the cohort will be enrolled through the Allergy and Asthma Federation (website and/or membership registry), a joint national organisation for patients suffering from asthma and other allergic conditions. All subjects to be enrolled must have a clinically confirmed diagnosis of bronchial asthma, based on the ICD-10 classification. The eligible patients must have the ICD-10 diagnosis of J45 (Asthma), including all the subgroups; J45.0 (Predominantly allergic asthma), J45.1 (Non-allergic asthma), J45.8 (Mixed asthma), or J45.9 (Unspecified asthma).

According to current recommendations for asthma RCTs, the subjects identified as having ''current asthma'' should fulfil the following criteria (98): 1) reported asthma diagnosed by a doctor and either symptoms of asthma or use of drugs for asthma in the previous 12 months, 2) show an increase in the forced expiratory volume in 1s (FEV1) >15% compared with baseline after bronchodilator administration, and/or 3) documented diurnal peak flow variation of >20% in any of the first 7 days of recordings.

3.1.2.Patients with other (non-asthmatic) conditions

Following the recommendations of the GINA guidelines (8,87), this proof-of-concept intervention trial shall exclude the participants with other related pulmonary diseases. These include (ICD-10 code): acute severe asthma (J46), chronic asthmatic (obstructive) bronchitis (J44.-), chronic obstructive asthma (J44.-), eosinophilic asthma (J82), lung diseases due to external agents (J60-J70), as well as status asthmaticus (J46). The exclusion of these non-asthma conditions strengthens the scientific robustness of this trial, which is designed to address the effects of L-cysteine on clinically established bronchial asthma only.

3.1.3.Level of asthma control

Before enrolment in the study, the level of asthma control of all potentially eligible subjects will be assessed using three validated classification tools: the Asthma Control Test™ (ACT)(95), the GINA guidelines (96) and the Asthma Therapy Assessment Questionnaire (ATAQ)(97). According to the latest GINA recommendations, the levels of asthma control include three categories: i) controlled, ii) partly controlled, and iii) uncontrolled. In the ACT, the cut-off between controlled and uncontrolled asthma levels off at score 19 or less (95), and using the ATAQ, score 1 out of 4 (max) should be the limit of alert (97).

To be eligible for the study, the subjects should present with **partly controlled** or **uncontrolled** asthma (95-97). This is because it is not reasonable to expect any observable therapeutic effects among the subjects whose asthma is under good control with the GINA Step 1 therapy (87). Clinically most relevant is to evaluate whether the new treatment brings any benefit for the patients with only partly controlled or uncontrolled asthma.

3.1.4.Duration of disease

Only subjects in whom asthma has been present for **at least 1 year prior** to entering into the study. This 1-year requirement helps excluding the subjects with i) probable (not fully confirmed) asthma, and ii) those with pulmonary symptoms showing features of asthma (J44 or J46, and others; see 3.1.2.). Furthermore, this minimum of 1-year course of established asthma improves the homogeneity of the study population.

3.1.5.Age at asthma onset

Subjects to be enrolled should report the onset of their asthma **before 50 years of age.** This is because i) true asthma with onset after 50 years of age is more rare (adult-onset asthma), and ii) there is increasing uncertainty in the diagnosis of true asthma among older people. This is simply because asthma symptoms can mimic other illnesses or diseases, especially in these older adults, including hiatal hernia, stomach problems or rheumatoid arthritis. Similarly, chronic obstructive pulmonary disease (COPD) has many of the symptoms in common with asthma, and the prevalence of COPD (emphysema and chronic bronchitis), is quite common in older adults, especially those who are or have been smokers (2,5,7,8,23,24,87,96).

3.1.6.Age at study entry

Most typically, asthma develops during childhood, and special guidelines have been launched by GINA to evaluate the control of childhood asthma (87,96). The same applies to the other assessment tools (ACT, ATAQ) as well, being different for childhood and adult asthma. Following these general guidelines, our cohort will accept only participants **between 18 and 65 years of age.**

3.1.7.Gender

Both **male** and **female** participants shall be eligible in the present cohort. Asthma has a higher prevalence in boys than in girls before puberty, but a higher prevalence in women than in men in adulthood. This contributes to the fact that no major gender imbalance exists among adult asthma patients. However, there are some key differences between the genders as related to development and progression of asthma (e.g. in genetic and immunological aspects), and it is recommended that all double-blind studies need to be **stratified by sex**, and treatment responses in females and males should be investigated separately (99)(see section 3.2.6).

3.1.8.Concomitant medication

All enrolled subjects will be allowed to **continue their current medication** for the management of asthma by GINA Steps 1-5 (87). This applies both to the maintenance therapy and the rescue therapy for acute asthma attacks (exacerbations). Because Acetium capsules have no known interactions with other drugs, also the other drugs not taken for asthma are not contraindicated.

Excluded are the following subjects: patients who have taken anti-psychotics or anti-depressant medications during the previous 3 months; patients who abuse alcohol or other drugs; and

potentially fertile and sexually active women who do not practise contraception.

3.1.9.Co-morbidity

The intention is to enrol a cohort of subjects with minimum co-morbidity. Specific co-morbid medical conditions that **exclude participation** in this trial include the following categories of patients: other acute or chronic pulmonary diseases, severe psychiatric disease, infections, malignancy, short life expectancy, cardiovascular disease, cerebrovascular disease, uncontrolled hypertension, degenerative central nervous system diseases, as well as pregnant and lactating women.

3.2.Trial design

This study design fulfils the criteria of a RCT accepted as Level A evidence in the Global Strategy for Asthma Management and Prevention by the **Global Initiative for Asthma** (GINA)(8,87).

3.2.1.Pre-trial observation period

Inherent to the special characteristics of asthma, a special pre-trial observation period is needed to assess the key baseline characteristics of the condition. In this trial, this is accomplished by a **3-month retrospective history** and **1-month prospective baseline (run-in)** period.

A detailed assessment of a 3-month retrospective history provides some assurance on the stability of asthma attack frequency prior to enrolment, but this should be confirmed prospectively by a 1-month baseline (run-in) period. Regularly, this 1-month prospective period provides more accurate data on asthma control than the 3-month retrospective period, because it minimizes the recall bias and allows for more precise determination of the mean attack frequency at baseline. In subjects with no attacks during the 1-month prospective run-in period, the baseline attack frequency must rely on the 3-month retrospective period.

3.2.2.Blinding

Following the most stringent recommendations for RCTs, this company-sponsored trial with Acetium capsules can be conducted in **triple-blinded fashion**; i.e., 1) participant-blind, 2) investigator-blind, and 3) sponsor-blind (=statistician evaluating the study results), to exclude the possibility of an undue bias on the results caused by data analysis.

3.2.3.Placebo control

Placebo preparation with design and package identical to the test preparation (Acetium capsule, 100mg) will be used in this trial, received by the other study arm of randomly allocated subjects.

3.2.4.Parallel-group design

Based on careful weighting of the advantages and drawbacks between the parallel design and the cross-over design, the current trial will be conducted as a **parallel group** design. Despite the undeniable advantages of the cross-over design in study power issues, its several important drawbacks contributed to the decision in favour of the parallel group design. Importantly, the fluctuating course of asthma poses similar challenges in both these study designs.

3.2.5.Randomization

Because patients are expected to be recruited to this prophylactic asthma trial over extended period, it is recommended to randomize in relatively small blocks, because participant selection may vary with time. In this trial, randomization will be performed after the 1-month prospective baseline (run-in) time, using a random number generator (https://www.sealedenvelope.com/simple-randomiser/v1/lists), with **blocks size of 4**, and creating **unique randomization codes** for each subject. The latter will be used as the only identifier of each subject in all datasets. Printed list (CSV Excel) is sealed in an envelope and stored in the company safety box, until opened at the completion of the study and all data analysis.

3.2.6.Stratification

Randomization alone may not ensure full comparability between participants in the two treatment arms, and stratified randomization is sometimes needed to remedy this potential imbalance between the two arms. Of the baseline asthma characteristics that potentially affect the efficacy outcomes, the prime candidate is gender. Indeed, there are some key differences between the genders as related to development and progression of asthma (including genetic predisposition and immunological aspects), and it is recommended that all double-blind studies need to be **stratified by gender** (99). Another option would be to control for the potential confounding by the gender in the primary statistical analyses, by including this variable as a covariate in the multivariate analyses (see Statistical Methods). It is also recommended that the treatment responses in females and males should be investigated separately.

3.2.7.Duration of the treatment period

Asthma is a chronic condition with protracted and fluctuating course, both posing challenges in the statistical modelling of its natural history. In asthma intervention RCTs, the primary efficacy endpoint is the attack frequency (asthma exacerbation days), and keeping the treatment periods relatively long is likely to provide **more stable estimates** of asthma exacerbation. Furthermore, only the **sustained effects** are clinically relevant, and these benefits clearly outweigh the potential risks of dropouts inevitably associated with longer treatment periods (99). In this trial, the **treatment period will be 3 months.**

3.2.8.Symptomatic (rescue) treatment

The optimal treatment for acute attacks in asthma prevention RCTs is both ethically and clinically indicated. In this trial, the participants should continue using their current treatment, because not anticipated to interfere with the study medication.

3.2.9.Follow-up visits

During the 3-month treatment period, participants will be evaluated **at monthly intervals** by the study coordinator, to review the diaries, monitor adverse events, ensure compliance and promote continued participation in the study. The follow-up will be concluded by the last clinical visit at the end of the 3-month treatment period.

3.2.10.Compliance

Evidence of poor compliance with asthma prophylactic RCTs is well established, and if poor enough, can be incorrectly interpreted as drug failure (98). Therefore, it is crucial to monitor patients' compliance with the test preparations during the entire study period. One such approach is the drug or pill count at every follow-up visit, and repeated emphasis on the values of adherence to the protocol requirements. The same applies to proper completion of asthma diaries.

Irrespective what measures are taken, it can be anticipated that the number of subjects lost to follow-up, those not completely adherent to the study protocol, as well as those interrupting the intervention for other reasons, will not be negligible in both study arms. It is likely that the final analyses must be run separately for two groups: **1) Per Protocol (PePr)**, and 2) **Intention-to-treat (ITT).** The former includes all subjects who have been compliant with the study protocol, without

any major violations in i) taking the test preparations, and ii) in recording their asthma diaries. The latter category includes all subjects who were not fully compliant with the protocol, but who completed all the follow-up visits and provided (at least) reasonable records of their diaries. There are opinions stating that statistical analysis of all RCTs should be done for the intention-to-treat (ITT) patient group only, so as to exploit the maximum amount of the recorded data (100).

4.METHODS

4.1.Baseline data

Before enrolment in the cohort, all subjects are requested to sign **a written consent**, after having been explained the details of the study and the commitment requested from each subject for the successful completion of their 4-month study protocol: 1-month run-in period, 3-month treatment period.

Before initiation of the 1-month prospective (run-in) baseline period, each subject will be requested to fill in a structured **Questionnaire** recoding their detailed asthma history, including the on-going medications (**ANNEX 1).** This Questionnaire also records the asthma attack frequency during the **3-month retrospective** period, immediately preceding the enrolment in the cohort, to be used as the baseline data in case that the 1-month run-in period does not yield any attacks.

4.2.Asthma diary and symptom scores

The asthma diary (**ANNEX 2**) is the main research tool used to monitor the efficacy of the test preparations on the control of asthma on daily basis throughout the entire study period. There are no specific recommendations about the design of such an asthma diary, other than it should be an easy-to-use, either a paper-and-pencil form or an electronic diary that captures all predefined assessment measures (efficacy, tolerability and safety).

In addition to the diary, all patients will be issued a validated diary card for recording daily **daytime** asthma symptom **scores** and **nocturnal** awakenings (**ANNEX 3**). Daytime symptoms are evaluated each night before bed, and night time symptoms are evaluated in the morning on rising, using validated measurement scales (101). The daytime asthma symptom scale uses a range of response categories for each question from 0 to 6, indicating the least to the most asthma symptomatology.

The nocturnal diary scale uses response categories ranging from 0 (indicating no awakening with asthma symptoms) to 3 (indicating awake all night).

Daily daytime scale scores will be computed as the average of the four questions on the daytime symptom scale (101). An overall diary score for the week will be computed as the average of the daily daytime scale scores. Weekly average scores for the nocturnal diary scale are computed in a similar manner. A decrease in the weekly score for the daytime and nocturnal scales indicates an improvement in asthma symptoms. The change from baseline in the asthma scale scores will be computed as the difference between the average score from the 1-month run-in period (or 3-month retrospective period) and the last two weeks of the active treatment phase.

4.3.Evaluation of asthma control

In addition to the asthma diary and the daytime and nocturnal scores, the effect of medication on the overall control of asthma will be assessed by comparing the baseline status with the post-treatment status, using three validated classification tools: the Asthma Control Test™ (ACT)(95), the GINA guidelines (96) and the Asthma Therapy Assessment Questionnaire (ATAQ)(97)(**ANNEX 4**). According to the latest GINA recommendations, the levels of asthma control include three categories: i) controlled, ii) partly controlled, and iii) uncontrolled. In the ACT, the cut-off between controlled and uncontrolled asthma is set at score 19 or less (95), and using the ATAQ, score 1 out of 4 (max) should be the limit of alert (97).

4.4.Spirometric evaluation

The assessment of the respiratory function is an essential part of asthma management. In this study, the patients will be examined by spirometry at three time points during the study: i) at enrolment, ii) at randomization, and iii) after completion of the treatment period. The routine procedures will be followed to measure the forced expiratory volume in one second (FEV1) and forced vital capacity (FVC), in a clinical setting.

4.5.Peak expiratory flow rate (PEFR)

As a part of the daily monitoring of asthma, patients are measuring their peak expiratory flow rate (PEFR) twice a day, using a mini-Wright peak flow meter (provided by the project), the values to be

stored in the attach diary (ANNEX 2). These PEFR measurement are done in the morning upon awakening and in the evening, before taking the evening dose of the study medication.

4.6.Study endpoints

The single most important aim of this study is to establish whether l-cysteine (Acetium capsule) is an effective measure in alleviating asthma exacerbation (=decreasing asthma attacks) among adult patients suffering from bronchial asthma. The null hypothesis of the study implicates that l-cysteine is no better than placebo in asthma prophylaxis during the intervention period of 3 months.

4.6.1.Primary endpoints

Rejection or not of the null hypothesis is based on comparison of the two strata (Acetium and placebo) against the **primary efficacy endpoint**. This pre-specified primary efficacy endpoint is **the percentage of asthma exacerbation days**, defined by Vaquerizo et al. (2003)(100) as the days when **any of the following occur**: i) awake all night (awake all night or recurrent episodes of awakening)(ANNEX 3: nocturnal scale), ii) increase of >50% from baseline in the symptom score (ANNEX 3: daytime scale), iii) increase from baseline in β-agonist use of >70% (minimum increase 2 puffs/day), iv) decrease from baseline of >20% in the morning PEFR, v) morning PEFR <180 l/min, or vi) an asthma attack (i.e., unscheduled medical care for asthma).

Two **alternative definitions** are available for calculating the primary efficacy endpoint (asthma exacerbation day). **First,** according to Wilding and co-workers (102) and by Chan-Yeung et al. (103), asthma exacerbation day is defined as a day when any of the following occur: i) an asthma attack or, ii) on two consecutive days, nocturnal waking, increase from baseline of more than 50% in symptoms score, use of at least 4 puffs/day of β–agonist, decrease from baseline of more than 30% or more than 100 l/ min in PEFR, or daily variability of more than 20% in PEFR (102,103). **Second** definition of an asthma exacerbation day was defined by Pauwels et al for (104). According to this definition, a day of **a mild exacerbation** is the one when any of the following occur: i) decrease from baseline of more than 20% in morning PEFR, ii) increase from baseline in β- agonist use of at least 3 puffs, or iii) nocturnal waking because of asthma. Importantly, single isolated days of exacerbations were not counted using this definition (104). In this study, we will use these alternative definitions in ad hoc analysis of the data.

4.6.2.Secondary endpoints

In addition to this primary efficacy endpoint, RCTs of asthma intervention recommend using different secondary endpoints: 1) **the number of asthma-free days** (100,102-104) is defined as a day when **all of the following occur**: i) no nocturnal waking, ii) use of two puffs or less of β-agonist, iii) no use of oral corticosteroids, and iv) no unscheduled use of medical care for asthma. Other pre-specified secondary endpoints are 2) the daily use of β–agonist and 3) asthma-specific quality of life. In addition, a variety of tertiary endpoints can be used, including morning PEFR, daytime symptom score, discontinuations secondary to asthma, FEV1, and patient and physician global evaluations. The latter include also the overall estimation of the asthma control, using the validated classification tools (ANNEX 4).

4.7.Statistical analysis

All statistical analyses will be performed using the SPSS 21.0.0 for Windows (IBM, NY, USA) and STATA/SE 13.0 software (STATA Corp., Texas, USA). The descriptive statistics will be conducted according to routine procedures. Frequency tables will be analysed using the χ2-test, with the likelihood ratio (LR) or Fisher's exact test for categorical variables. Differences in the means of continuous variables are analysed using non-parametric (Mann-Whitney or Kruskal-Wallis) test for two- and multiple independent samples, respectively.

4.7.1.Conventional techniques

Standard statistics are used to compare the efficacy of the two study arms on asthma exacerbation (primary and secondary endpoints). The effects of test preparation and placebo can be analysed separately in non-parametric paired samples t-test (Wilcoxon signed ranks test) to compare the pairs of the baseline- and follow-up frequencies (asthma-attacks, asthma-free days) in both arms. Another way is to calculate the efficacy measures (reduction in the baseline attack frequency by treatment) and compare these effects between the two arms. Similarly, the statistics used to compare the two arms with regard to the secondary endpoints is straightforward.

4.7.2.Life-table techniques

The proportions of asthma exacerbation days and asthma-free days are calculated from the total number of follow-up days in each treatment group. From these proportions, we can calculate the absolute risk reduction, relative risk, reduction in relative risk, and their corresponding 95%CI.

The number needed to treat (NNT) can be obtained for each end point (asthma exacerbation and asthma-free days). The NNT is defined as the number of treatment days with Acetium needed to **prevent** either an i) asthma exacerbation day or ii) a non-asthma-free day (i.e., to gain an asthma-free day).

More sophisticated approaches can be used to analyse these longitudinal data, pending on the obtained results. If, e.g. complete and sustained abortions of asthma exacerbation are recorded, life-table techniques can be used to compare the two study arms, using univariate survival (Kaplan-Meier) analysis, where the attack disappearance dates represent the event of interest, and the stratum-specific (test vs. placebo) estimates are calculated using the log-rank (Mantel-Cox) statistics. The same approach can be used to calculate the difference in the duration of attack abortion (i.e., consecutive asthma-free days) between the two study arms. The independent effect of Acetium (adjusted for potential confounders) can be analysed using the multivariate Cox proportional hazards regression models, where all recorded baseline characteristics of asthma (Questionnaire) can be entered as covariates, including the stratification variable (gender).

4.7.3.Generalised linear models (GEE, panel Poison)

In addition, using the "permanent" attack disappearance (abortion) as the event, the effect of Acetium versus placebo can be modelled also using the regression techniques based on count variables, i.e., Poisson regression. In this approach, attack disappearances are expressed as **events per person time (days) at risk**, and the two arms can be compared using the **incidence rate ratio** (IRR) statistics. When applied to panel type of data recorded each day (panel Poisson), all covariates subject to random intra-subject variation (by day) can be adequately controlled, in this longitudinal setting. A similar type of approach based on panel data, i.e., generalized estimating equation (GEE) modelling, can be used to estimate the effect of Acetium/Placebo on persistence (=sustainability) of attack remission (=asthma-free days, AFD), using the AFD (yes/no) recorded at each day as the dependent variable, and adjusted for potential confounders in multivariate GEE. A wide variety of such potential confounders should be examined, including e.g. age, gender, asthma type, asthma onset, severity of asthma, attack frequency, asthma control, triggers, etc.

4.7.4.Modelling of migraine outcomes by competing-risks regression

In all study settings where repeated measures involve the same subjects, the results tend to be

correlated. In other words, the probability of recording an asthma attack is greater among subjects who reported an attack (or no reduction in attack frequency) in the previous record, if the record is repeated within a reasonable time (in this case on daily basis). Statistical techniques that i) fail to take these correlations into account are not valid, and ii) methods that do not exploit all the recorded data (in a repeated measures setting) would be inefficient. Marginal (GEE, Panel Poisson)- and mixed-effects models are both capable of handling these issues, showing a greater efficiency as compared with standard logistic regression and Cox models for studying the outcomes of migraine intervention. Both methods are technically suitable for analysis of the panel data of the present study setting, but because they only accept a binomial (yes/no) dependent variable, this would necessitate a separate analysis for each of the multiple study outcomes (see the following).

Asthma prevention trial is more complex than merely showing a reduction in exacerbation days or increase in asthma-free days, vs. no such reductions, as dichotomous (yes/no) outcome. Indeed, it can be anticipated that there are several possible outcomes to be observed during the Acetium intervention, which can be treated as **competing events.** These include the following: **i) no effect** (=exacerbation and/or asthma-free days remain unchanged as compared with the baseline), **ii) abortion** of exacerbations (=disappearance of all asthma episodes since the onset of intervention), **iii) relapse** (=disappearance of exacerbations for a period of time but subsequent reappearance of the attacks), and **iv) reduction** of exacerbations (=exacerbations are reduced at study endpoint, but not entirely aborted).

If the i) the longitudinal data be utilized in full, ii) intra-subject variation of the repeated measurements be taken into account, and iii) the multiple-outcome dependent variable (no effect, abortion, relapse, reduction) be treated in a single statistical model, we need to apply a special technique, known as **competing-risks regression** (105,106). This elegant technique can be used to model the impact of Acetium intervention on the competing risks outcomes of this trial, adjusted for other covariates as potential confounders.

4.8.Power analysis

Due to the fact that several optional tools are available for statistical analysis of these data, also the power of the study can be analysed differently, following the algorithms specified for each of these statistical techniques. In the simplest approach (reduction of exacerbation days by treatment), the

power can be calculated using the two-sample proportion test, comparing the proportion of exacerbation days (or their reduction) in the Acetium and placebo arms. The study (n=100 per study arm) is adequately powered (Type II error 0.80, type I error 0.05) to detect a true difference in proportion of exacerbation days between 0.40 in the placebo and 0.22 in the Acetium arm, i.e., the difference in effect size of 18%. Within this effect range, the study power is sensitive to any decrease in this difference and also critically dependent on the SD in the two arms. Given that the study subjects are selected among patients with partly controlled or uncontrolled asthma, these figures seem reasonable estimates for the basis of these power calculations, however (100).

5.STUDY EXECUTION AND TIME TABLE

For execution of the study, the company has decided to set up and monitor the whole study in their own premises. For this purpose, the company recently hired a project coordinator, specifically devoted to this study. She will take care (with the principal investigator) of all the necessary practical issues, starting from invitation of volunteer asthma patients (with assistance of Allergy and Asthma Federation), their first interview (with spirometry) at study entry, delivery and return of the asthma diaries for the 1-month run-in period, randomization of the subjects for study arms, delivery of the numbered packages of the test substances, follow-up visits on monthly bases, collecting the asthma diaries, and finally the transfer of all collected data into the data file.

The study protocol will be circulated among the Scientific Board Members of Biohit Oyj, as well as subjected to inspection by the consultant expert pulmonologist. As soon as possible, the final protocol will be subjected for approval by the National Committee on Medical Research Ethics (TUKIJA). Meanwhile, preparatory measures will be initiated, including the contacts with expert pulmonologist to be involved in the study, contacts with potential partner clinics needed to examine the patients at baseline and at study conclusion, as well as exploring the possibilities to get the AAF involved. The idea is that AAF could assist us in the process of inviting asthma patients who are interested in participating to contact the study coordinator. Given the preliminary interest shown by the AAF, we are optimistic that the required cohort of volunteers will be available within a short period since the completion of the formalities for Ethical Committee approval, or during the first quarter of 2014. Given that each study subject shall complete the 3-month treatment period, preceded by 1-month run-in time, we expect that the study will be completed during the second half of 2014.

6.PROJECTED COSTS

All project costs will be covered by the sponsor, Biohit HealthCare, Oyj. Stratified research budget is possible only when all cost categories are known.

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ANNEX 1. **ASTHMA HISTORY RECORD**

Date of Interview:	Day:	Month:	Year:	
Name:				
Date of Birth:	Day:	Month:	Year:	AGE:
Gender:	Female:		Male:	
Profession:				
Chronic co-morbidities	Yes:		No:	
If yes, list the most important ones:				
Asthma diagnosis made:	Year:		Age at onset:	
The exact diagnosis (ICD-10 code):	**J45.0:** Predominantly allergic asthma:		Bronchitis NOS	
	Rhinitis with asthma		Atopic asthma	
	Extrinsic allergic asthma		Hay fever asthma	
	J45.1: Non-allergic asthma:		Idiosyncratic asthma	
	Intrinsic non-allergic asthma			
	J45.8: Mixed asthma:		Combination of conditions in J45.0 and J45.1	
	J45.9: Unspecified asthma:		Asthmatic bronchitis NOS	
	Late-onset asthma			
Symptoms of asthma (typical attack):	Early warning signs (Yes):		No:	
	Coughing at night:		Losing breath:	
	Shortness of breath:		Getting tired:	
	Feeling weak after exercise:		Symptoms of cold:	
	Sneezing:		Sore throat:	
Early warning signs:	Frequency (%):	Timing (h before):	Main symptom:	
Associated conditions:	Yes:		No:	
	GERD (reflux disease):		Rhinitis-sinusitis:	
	Obstructive sleep apnea:		Psychological disorders:	
	Other allergies:		Hypersensitivity conditions:	
Causes and Triggers:	Genetic background (Y/N, details):			
Environmental (describe)				
Medical conditions (describe)				
	Tobacco smoke:		Pets:	
	Bugs:		Fungi:	
	Strong emotions:		Air pollution (outdoor):	
	Weather:		Alcohol:	
	Foodstuffs:			
Frequency of attacks (current):	No./Day:	No./Week:	No./Month:	More rarely:
Trend since the asthma diagnosis	Constant:	Increased:	Decreased:	
	Frequency of attacks during the past 3 months (IMPORTANT)			
	No./Day:	No./Week:	No./Month:	No attacks:
Preventive medical treatment:	YES:		NO:	
	RABA:		ICS:	
	LABA:		LTRA:	
	MAB:		Other:	
Current preventive treatment:	List the medicines:			
	1	2	3	
	4	5	6	
Current treatment of acute attacks:	List the medicines:			
	1	2	3	
	4	5	6	
Your self-estimation of your asthma:	Under good control:		Not in satisfactory control:	
	Improved during the years:		Aggravated during the years:	
	Debilitates my daily life:		I can live with it:	

SOME OF YOUR LIFE-STYLE PRACTICES					
Alcohol consumption:					
Regularity and type	No:	Social:	Daily:	Excessive:	
Type of alcohol typically used	Beer:	Wine:	Liquors:	Spirits:	Other:
Weekly use	Estimated dosages per week:				
Alcohol intake triggers your asthma?	NO:		YES:		
Did you stop drinking due to asthma?	NO:		YES:		
Smoking history:					
	Never:	Past:	Current:		
Age when started smoking					
Regular smoker ever since	Yes:	No:			
If not, describe					
Cigarettes per day (currently)					
The same number, for how long (yrs)?					
Trend in the daily numbers of cigarettes	Constant:	Increasing:	Decreasing:		
Other forms of tobacco	Cigars:	Pipe:	Smokeless:		
If any of the above, list the amounts					
Smoking triggers your asthma?	NO:	YES:			
Did you stop smoking due to asthma?	NO:	YES:			
SPACE FOR FREE COMMENTS:					

ANNEX 2. **ATTACK DIARY FOR ASTHMA PATIENTS**

MONTH:	**SUBJECT CODE:**

Day	**Attack Y/N**	**Timing of the Attacks**		**Total Number of Attacks per 24h (D+N)**	**Acute Medicine (β-agonist) (Y/N)**	**How many puffs of AM?**	**Your satisfaction on the test drug#** (1-5)	**PEFR**		**Side effects ascribed to the test drug:** (see footnote for coding)		
		Day	**Night**					**M**	**E**	**ES** (1-3)	**ESR** (1,0)	**D** (h)
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
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31												

OVERALL EVALUATION OF YOUR ASTHMA MONTH

Days	No. attack Days	Total Day	Total Night	Total No. Attacks	AM Used (Y/N)	No. AM Puffs Used	Average Score		No. of Side Effects	Total D

*__AM__ (Acute Medicine= β-agonist); **#Satisfaction**: (1=very dissatisfied; 2=somewhat dissatisfied; 3=neither satisfied nor dissatisfied; 4=somewhat satisfied; 5=very satisfied); **PEFR**, peak expiratory flow rate; **M**, morning; **E**, evening; **SIDE EFFECTS**: 1. Event severity **(=ES)**: (1=mild, 2=moderate, 3=severe); 2. Event seriousness (=**ESR**): (1=serious, 0=non-serious); 3. Duration **(=D)**: (hours).

ANNEX 3. **DAYTIME AND NOCTURNAL SYMPTOM DIARY**

Daytime symptom diary scale questions

1) How often did you experience asthma symptoms today?

0	1	2	3	4	5	6
None of the time						All of the time

2) How much did your asthma symptoms bother you today?

0	1	2	3	4	5	6
Not at all bothered						Severely bothered

3) How much activity could you do today?

0	1	2	3	4	5	6
More than usual activity						Less than usual activity

4) How often did your asthma affect your activities today?

0	1	2	3	4	5	6
None of the time						All of the time

Nocturnal diary scale question

1) Did you wake up with asthma symptoms? (This can be awakening in the middle of the night or on awakening in the morning).

❑ No	❑ Once	❑ More than once	❑ Awake "all night"
0	1	2	3

ANNEX 4. **THE 3 VALIDATED ASTHMA CONTROL CLASSIFICATION TOOLS**

1) The revised GINA criteria (2006)

Characteristic	**Controlled**	**Partly controlled**	**Uncontrolled**
Daytime symptoms	None (or minimal)	More than twice/week	Three or more features of partly controlled asthma present in any week
Limitations of activities	None	Any	
Nocturnal symptoms/ awakening	None	Any	
Need for reliever/rescue treatment	None (or minimal)	More than twice/week	
Lung function (PEF or FEV_1)	Normal or near normal	<80% predicted or personal best (if known) on any day	
Exacerbations	None	One or more/year*	One in any week**

*Exacerbations occurring more than once a year should prompt review of maintenance treatment to ensure that it is adequate. **By definition, an exacerbation in any week makes that an uncontrolled asthma week.

2) Asthma Control Test (ACT™)

1. In the past 4 weeks, how much of the time did your asthma keep you from getting as much done at work, school or at home?

SCORE

All of the time	1	Most of the time	2	Some of the time	3	A little of the time	4	None of the time	5	

2. During the past 4 weeks, how often have you had shortness of breath?

SCORE

More than once a day	1	Once a day	2	3 to 6 times a week	3	Once or twice a week	4	Not at all	5	

3. During the past 4 weeks, how often did your asthma symptoms (wheezing, coughing, shortness of breath, chest tightness or pain) wake you up at night or earlier than usual in the morning?

SCORE

4 or more nights a week	1	2 or 3 nights a week	2	Once a week	3	Once or twice	4	Not at all	5	

4. During the past 4 weeks, how often have you used your rescue inhaler or nebulizer medication (such as albuterol)?

SCORE

3 or more times per day	1	1 or 2 times per day	2	2 or 3 times per week	3	Once a week or less	4	Not at all	5	

5. How would you rate your asthma control during the past 4 weeks?

SCORE

Not controlled at all	1	Poorly controlled	2	Somewhat controlled	3	Well controlled	4	Completely controlled	5	

If your score is **19 or less**, your asthma may not be under control. TOTAL SCORE _____

References:

1. US Department of Health and Human Services, National Institutes of Health, National Heart, Lung and Blood Institute. Expert Panel Report 3: Guidelines for the Diagnosis and Management of Asthma (EPR-3 2007). NIH Item No. 08-4051. http://www.nhlbi.nih.gov/guidelines/asthma/asthgdln.htm. 2. Nathan RA et al. J Allergy Clin Immunol. 2004;113:59-65.

3) Adult (18 years) Asthma Therapy Assessment Questionnaire (ATAQ)

1. In the past 4 weeks, did you?

Miss any work, school, or normal daily activity because of your asthma?	YES 1	NO 0	Unsure 1	Score:
Wake up at night because of asthma?				
Feel your asthma was not well controlled?				
2. Do you use an inhaler for quick relief from asthma symptoms?	Yes	No	Unsure	xxxxxxxx

(**If Yes**) In the past 4 weeks, what was the greatest number of puffs in one day you took of the inhaler?

0 puffs	0	9 to 12 puffs	0
1 to 4 puffs	0	More than 12 puffs	1
5 to 8 puffs	0	Score	

TOTAL SCORE _____

If your total score is **1 or more**, your asthma may not be in good control