An 18 year Follow-up of Allergy Development –
Findings of Nasal Markers of Allergic Inflammation

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Potius sero quam numquam
ABSTRACT

**Background:** In addition to the family history of allergy (FH), there is a need to find objective markers of allergy development as early in life as possible in order to focus preventive measurements on high risk infants. Rhinitis problems are common causes to morbidity in adults due to allergic as well as non-allergic mechanisms. Accurate diagnoses are essential for decisions of optimal management of the patients, but in non-allergic rhinitis groups there are no objective tests to verify the diagnosis, if this is needed.

**Aims:** The primary aim was to evaluate the occurrence of nasal metacromatic (MC) cells during infancy as predictors for allergy development in a group of high risk subjects from birth up to 18 years of age. Additional aims were to find and evaluate nasal markers with ability to differentiate between allergic rhinitis with and without current allergen exposure from normal controls.

**Subjects and methods:** New-borns (n = 67) with and without family histories of allergy were included, and during the first 18 months of life occurrence of nasal MC could be evaluated in 64 infants (33 positive/31 negative MC findings). The cohort was followed up for allergy development at the ages of 18 months, 6 years and 18 years. Nasal markers as MC, nasal NO, nitrite/nitrate in nasal lavage and acoustic rhinometry at the 18-years follow-up were related to the allergic manifestations at this age.

**Results:** Positive nasal MC findings during infancy predicted allergy development up to 18 years of age in 31/33 subjects (94 %), as compared to 37/44 with positive FH (84 %). Negative MC findings during infancy did not exclude the risk, as 15/31 developed allergy (48 %). At the 18-years follow-up the numbers of individuals with demonstrable MC were significantly higher (p = 0.01) in the group of individuals with allergy symptoms (16/30) compared to the group of individuals with no allergy (1/12). Nasal NO levels, nitrite/nitrate concentrations in nasal lavages and acoustic rhinometry did not differentiate the allergic groups from the normal group.

**Conclusions:** Positive nasal MC findings during infancy predicted allergy development up to 18 years of age, and the cell findings often preceded the allergic symptoms. The marker can not be used as a single predictor of allergy development due to negative MC findings in a high proportion of allergic subjects. Positive MC findings combined with positive FH resulted in the best the risk evaluation. Differences between groups with and without current allergen exposure and healthy controls were not found by means of acoustic rhinometry, nasal MC, nasal NO or nitrites/nitrates levels. Further research to find reliable nasal markers is needed.
LIST OF PUBLICATIONS

I  Nasal metachromatic cells in infancy in relation to the appearance of atopic disease during the first 6 years of life. Borres MP, Irander K, Björkstén B. 
Allergy 1997; 52: 770-774.

II  An 18 year follow-up of allergy development related to nasal metachromatic cell findings during infancy. Irander K, Borres MP. Submitted

III  Nasal nitric oxide and nasal nitrates and nitrites in relation to allergy and smoking habits. Irander K, Palm J, Borres MP. In manuscript
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ARC</td>
<td>allergic rhinoconjunctivitis</td>
</tr>
<tr>
<td>CC16</td>
<td>clara cell protein 16</td>
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<tr>
<td>FH</td>
<td>family history of atopy</td>
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<tr>
<td>MC</td>
<td>metachromatic cells: mast cells and basophils</td>
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<tr>
<td>NAL</td>
<td>nasal lavage</td>
</tr>
<tr>
<td>NARES</td>
<td>non-allergic rhinitis with eosinophilia syndrome</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>- nNO</td>
<td>nitric oxid from the upper airways</td>
</tr>
<tr>
<td>- eNO</td>
<td>nitric oxid from the lower airways</td>
</tr>
<tr>
<td>ns</td>
<td>not significant</td>
</tr>
<tr>
<td>PLUNC</td>
<td>palate lung nasal epithelial clone</td>
</tr>
<tr>
<td>RAST</td>
<td>radio allergo sorbent test</td>
</tr>
<tr>
<td>SPT</td>
<td>skin prick test</td>
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<tr>
<td>VOL2</td>
<td>the volume in the nasal cavity between 2.20 cm and 5.40 cm from the nostril</td>
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INTRODUCTION
Atopic diseases are common problems world-wide in all age groups (1, 2). The natural history of atopic manifestations is described as “the atopic march” (3), starting with sensitization to food allergens and atopic eczema during the first 1-2 years of life. As the problems with atopic eczema and food allergy gradually grow out, there is a risk of sensitization to aero-allergens, especially in children with early allergy debut, resulting in development of respiratory allergy with asthma and rhinitis often in combination with conjunctivitis (4-7).

Rhinitis problems are classified into three main groups: infectious rhinitis due to infectious agents, allergic rhinitis due to IgE mediated reactions, and the heterogeneous group of non-allergic rhinitis, which includes all of the rhinitis problems without grounds of immunological or infectious mechanisms (8, 9).

All of the rhinitis groups may occur separately or in various combinations (10). During childhood and adolescence the most common cause of the rhinitis is allergy beside infections, but with increasing age different forms of non-allergic rhinitis are often added. Rhinitis problems, allergic as well as non-allergic forms, are significant causes of morbidity and decreased quality of life (11-14). Defining the accurate diagnoses is an essential base for decisions of optimal management in the affected individuals. A careful medical history including the relation between symptoms and exposure to offending agents and a careful physical examination of the nose are the bases in diagnosing, but complementary objective tests might be needed.

Diagnosis and prediction of allergy
Atopy is regarded a systemic disease with manifestations in different organs (15, 16). Diagnosis is based with good accuracy on the history of allergy symptoms, a physical examination and, when needed, objective tests by demonstration of allergen-specific antibodies in serum by RAST or in the skin by a SPT. Intranasal challenge tests with allergens or non-specific agents are primarily used in research studies, and usually not needed in clinical practice and (17-19). Asthma diagnosis is evaluated by spirometry, peak expitatory flow (PEF) measurements, and if available in the clinical practice, by measurements of exhaled nitric oxide.
An important issue in the management of atopy is the prediction and identification of risk factors of allergy development, as preventive measurements have to be focused on individuals with high risk in order to delay and attenuate their symptoms (20-22).

The family history of asthma and atopy (FH) is regarded as one of the most important predictive factors (23-25). However, not all of the subjects with a positive family history develop allergy, and all subjects with a negative family history do not remain healthy from allergy problems (26). Although extensive studies of the human genome have given much information about the genetics of allergy diseases (27), genetic markers are not yet available in clinical practice.

Many studies have documented environmental risk factors, such as exposure to tobacco smoke (28, 29), but studies on exposure to furry pets have given controversial results (28, 30).

Understanding the immunological mechanisms underlying inflammation has identified a number of biomarkers, but few of them have proven useful as predictors (21, 31). Thus, there is a need to find biomarkers with high predictive accuracy for allergy development and to find the markers as early in life as possible in order to optimize preventive measurements.

**Diagnosis of non-allergic subgroups**

Non-allergic rhinitis comprises many subgroups with diagnoses according to the history of symptoms and their relation to offending agents as air-born pollutants in general or to occupational irritants, pharmacological drugs, beverages, hormones, emotions, physical factors, or according to histological findings as atrophic, eosinophilic (NARES) or as idiopathic without any relations (9-13).

The pathophysiology is poorly understood or un-known. No specific tests are available in any of the subgroups. Functional tests, such as acoustic rhinometry or rhinomanometry, do not give enough information by occasional measurements in single subjects due to extensive individual variations; however, these methods identifies mucosal changes at repeated measurements in the same individuals (32, 33). This is in contrast to spirometry with defined limits between normal and abnormal values (34). Nasal provocation tests with occupational allergens, although laborious, might be performed (35), but intranasal challenges with metacholine, histamine or capsaicin, which have good reproducibility in the lower air-ways, are mainly used in experimental research of the upper air-ways but not in clinical practice (36, 37).
The lack of objective methods identifying non-allergic rhinitis groups is a great shortcoming in situations, where contacts with authorities demand objective tests before proper measurements can be taken, an aggravating problem e.g. in airway diseases related to occupational environments.

**Diagnostic needs of rhinitis markers in clinical practice**

Thus, there is a need to find intranasal markers or a profile of markers, which can differentiate between normal, allergic and at least some of the non-allergic rhinitis subgroups. The nasal cavity is easily available for clinical examination, challenge tests, functional test and sampling of materials for analyses of cells and markers in the secretion. When comparing marker concentrations between different rhinitis groups, it is important that the groups are well defined. Allergic rhinitis is the most easily defined group.

In 1985 a cohort study was started aiming to find early predictors of allergy development (38). The main focus in the cohort study was on the occurrence of metachromatic cells (MC), in the nasal mucosa, as new information on human mast cell heterogeneity (39) had been recently available at the study start. The method used for visualization of mast cells does not separate mast cells from basophils, the other metachromatically staining cell, but it was the only method available at that time.

The relation between nasal MC findings and development of allergy was followed-up at the age of 18 months (38), and a second follow-up was performed at the age of six years. When subjects were invited at the age of 18 years to a third follow-up of their allergy problems, they were asked to participate in tests for nasal markers. This group of teenagers with well-defined allergic disorders, still unaffected by occupational irritants, and with good co-operative ability in various tests, were regarded as an excellent group for analysis of nasal markers, provided considerations were taken to passive smoke exposures and active smoking habits.
AIMS OF THE STUDY

• The original aim at the start of the cohort study was to evaluate the predictive value of nasal MC findings during infancy and development of allergy, with a follow-up at 6 years (Paper I) and at 18 years of age (Paper II).

• Additional aims at the 18 years follow-up were to analyse the ability of markers and functional tests to differentiate between subgroups of allergy, with and without current allergen exposure, and normal controls:
  - nasal MC_{18\text{ years}} (Paper II)
  - nasal NO and oral NO levels
  - nitrites/nitrate concentrations in NAL (Paper III).
  - acoustic rhinometry and spirometry
  - PLUNC findings in NAL and cells (analyses in progress)
  - CC16
SUBJECTS AND METHODS

Subjects

After informed consent, the invitations to participate in the study were accepted by 67 families (40 female and 27 male new-borns). In order to get a cohort of children in whom 50% would be expected to develop symptoms of atopy early in life (26), invited families with a high risk of allergy development in their infants (positive FH; n = 46) were in majority compared to low risk infants (negative FH; n = 21). After verifying the FH by SPT in the family members, as described previously (38), the study group was found to include 46 children with positive FH, 11 with possible and only 10 children with FH.

The 6-years and the 18-years follow-ups were performed during winter time out of pollen seasons. The participants had to be free from airway infections for at least 10 days prior to the tests.

A history of allergy development could be evaluated through interviews and questionnaires in all of the 67 children at both the 6-years and the 18-years follow-ups. All of them did not attend the clinic for tests, a few of the attending subjects refused to participate in single tests, and subjects using pharmacological agents affecting results were excluded in the evaluations. Numbers of subjects with evaluable results at the two follow-ups are shown in Table 1.

<table>
<thead>
<tr>
<th>Paper number</th>
<th>Aims in the study</th>
<th>Number of evaluable subjects</th>
<th>Number of subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>(follow-up at the age of)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I (6 years)</td>
<td>Allergy development</td>
<td>67</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>MC 6 years $\leftrightarrow$ atopy $\leq$ 6 years</td>
<td>54</td>
<td>7 / 0 / 6 = 13</td>
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<tr>
<td></td>
<td>MC 1-12 months $\leftrightarrow$ atopy $\leq$ 6 years</td>
<td>64</td>
<td>0</td>
</tr>
<tr>
<td>II (18 years)</td>
<td>Allergy development</td>
<td>67</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>MC 18 years $\leftrightarrow$ atopy $\leq$ 18 years</td>
<td>45</td>
<td>18+2 / 2 / 0 = 22</td>
</tr>
<tr>
<td></td>
<td>MC 1-18 months $\leftrightarrow$ atopy $\leq$ 18 years</td>
<td>64</td>
<td>0</td>
</tr>
<tr>
<td>III (18 years)</td>
<td>nNO/ac rhino$\leftrightarrow$ ARC / healthy</td>
<td>40</td>
<td>18 / 5 / 4 = 27</td>
</tr>
<tr>
<td></td>
<td>eNO/spiro $\leftrightarrow$ AB / helathy</td>
<td>43</td>
<td>18 / 4 / 2 = 24</td>
</tr>
</tbody>
</table>

Table 1. Numbers of subjects with evaluable results in the follow-ups. ARC = allergic rhinoconjunctivitis; AB = allergic bronchitis
**Questionnaires**

The questionnaires included detailed questions about symptoms in the upper, the lower airways and in the skin, offending allergens in air or foods, the need of pharmaceutical agents, exposure to pets, passive exposure to tobacco smoke and own smoking habits, the air quality at home or in school buildings, and a few questions about quality of life.

**Definitions of allergy diagnoses**

All allergy diagnoses were based on the history of allergy symptoms, reported in the questionnaires and completed, if needed, by interviews. Results from allergy tests were not included. Findings at the physical examination of the upper airways, auscultation of the chest and inspection of the skin were complementary in the diagnosis.

*Allergic rhino-conjunctivitis* definition was based in:

Paper I – III (6-years and 18-years follow-ups): on the reports of rhinitis symptoms (nasal itching, secretion, sneezing, nasal obstruction) and conjunctivitis (itching, redness of the eyes) at least twice after exposure to a relevant air-born allergen and not related to an infection.

*Asthma* definition was based in:

- Paper I (6-years follow-up) on a history of recurrent episodes of bronchial obstruction, verified at least once by a physician, in combination with a reduction in forced expiratory volume at 1 s (FEV1) of ≥15% after a simple running test; the same medical history with a reduction of < 15 % was classified as *probable asthma*.

- Paper II - III (18-years follow-up): on a history of bronchial obstruction (expiratory distress, wheezing) in relation to exposure to a relevant air-born allergen in combination with significant changes in the spirometric results in the exercise provocation test (see below). If the results did not reach significance, the symptoms were classified as *allergic bronchitis*. Bronchial symptoms related to infections and physical exercise but never to allergen exposures were classified as *non-allergic bronchitis*.

*Atopic eczema* was defined in:

- Paper I (6 years follow-up) according to the classification by Hanifin & Rajka (40)
- Paper II and III (18-years follow-up) according to the SCORAD definitions (41).
Probable airway allergy was defined on a history of allergy symptoms not fulfilling all of the criteria.

Recovery from the allergic disease was defined as absence for the last 2 years of symptoms, which previously were evoked by exposure to offending allergens.

Skin prick tests
Allergen extracts used in:
- Paper I: egg and milk (Dome/Hollister-Stier/Bayer extracs 1:20 w/v), fish (Dome/Hollister-Stier/Bayer extract 1:10 w/v), and animal epithelia, timothy, D. farinae, Cladosporium and birch (ALK, Hörsholm, Denmark).
- Paper II and III: birch, timothy, mugwort, horse, cat, dog, D pteronyssinus, D farinae, Alternaria, Cladosporium, hazel nut, almond and peanut (ALK Sverige AB). ImmunoCAP® analyses with the corresponding aero-allergens were used in one male with allergy including severe atopic eczema.

A SPT was defined as positive, if the mean wheal diameter (half of the sum of the largest diameter and its perpendicular) was at least 3 mm. Histamine hydrochloride 10 mg/ml served as a positive reference. Normal serum albumin was used as a negative control.

Acoustic rhinometry
Acoustic rhinometry in Paper III was performed using Rhin 2000 (S.R. Electronics A.S., Lynge, Denmark). The mean values from three recordings and the sum from both nasal cavities were calculated using the computerized program with predetermined calculations of minimal nasal cross-sectional areas and volumes (42). The volume between 2.20 and 5.40 cm from the nasal aperture the (VOL2), was used to report the results before and after exercise, as this parameter was regarded to best reflect mucosal changes in the nasal patency, an opinion also found by others (43).

Spirometry and the exercise provocation test
A method based on the GINA guide of Childhood asthma diagnosis (34) was used to evaluate the function in the lower airways. The individuals had to run on a treadmill for 6 minutes to achieve a pulse rate of ≥ 160 beats per minute. Spirometry (Microlab) was performed before exercise, immediately after exercise and repeated after another 15 minutes; a reversal dose of
a β-agonist was then given followed by a fourth measurement 15 minutes later. Asthma definition was based on the values of the forced expiratory volume in 1 second (FEV₁): an initial FEV₁ value ≤ 80% of the expected value, a reduction of FEV₁ ≥ 15 % after the physical exercise in combination with an increase of FEV₁ ≥ 15 % after the bronchodilatation.

**NO measurements from the upper and lower airways**

Measurements of NO in Paper III were performed using NIOX® (Aerocrine AB, Sweden). The nNO and eNO values were calculated according to the method described by Palm et al (44). Briefly, a tight-fitting facial mask was adapted on the mouthpiece when measuring nasally exhaled NO, and the mean of three recordings was calculated. Nasal measurements were followed by a mouthwash, using 20 ml of sodium bicarbonate solution (10%) for 1 minute, immediately followed by oral measurements (eNO), and the mean from three recordings was calculated. The contribution of NO from the upper airways (nNO) was calculated by subtracting the mean eNO value from nasally exhaled mean NO values.

**Nasal lavage**

Nasal lavage was performed by using saline, pre-warmed to 37⁰ C. The subject held the head bent forward with the face horizontally, while the left nasal cavity was filled with the saline using a syringe connected to the nostril via a short tube and a nasal olive. After five minutes the saline was aspirated back. After immediate centrifugation the supernatant was frozen (-20⁰ C) until analysis.

**Nitrite/nitrate analysis**

Nitrite and nitrate concentrations were analysed by the Greiss reaction after reduction of nitrate to nitrite according to the method described by Verdon et al (45).

**Cytospin preparations of nasal mucosal cells**

Nasal mucosal cells were harvested from the right nasal cavity by a gentle nasal brushing using a 5.5 mm diameter nylon brush (Doft AB, Östhammar, Sweden). The brush was immediately placed in a tube containing physiological saline and twirled for 3-5 seconds. After cytocentrifugation onto glass slides, the materials were air-dried and fixated in 95 % ethanol for later staining with toluidine blue for visualization of MC according to the method used in our previous follow-up (38). Analysis of the cell numbers of was performed by light microscopy (magnification x 250) with slides coded.
Markers evaluated in Papers I - III

Metachromatic cells

In Paper I and Paper II the value of nasal MC during infancy was evaluated as predictors of allergy development up to 6 and 18 years of age, respectively.

In Paper II the value of nasal MC_{18\text{ years}} was analysed as a marker with ability to differentiate between subgroups of allergic rhinitis, with or without current allergen exposures, and healthy controls.

Mast cells have been intensively studied during the last two decades. Mast cell progenitors are derived from the bone marrow, migrate into the blood stream and subsequently into all types of tissues, where the cells undergo their final stages of maturation into subsets containing tryptase, chymase or both of the proteases (46, 47). Mast cells are most common in sites exposed to external pathogens, such as the skin, the airways and the intestinal mucosa (47). In symptomatic ARC patients, mast cells containing both of the proteases were dominant in numbers (48).

Mast cells are studied not only in relation to allergy diseases, the cells are also found to be of great importance in many other diseases, as in wound healing, fibrosis and autoimmune diseases (46), in the host defence against parasites and bacterial and viral infections through both innate and acquired immune responses (47, 49) and in tumour growth (50).

In the follow-up of our cohort group at the age of 18 months, the relation between MC_{3-18\text{ m}} and middle ear diseases during the same period was performed as a part of all evaluations. Middle ear morbidity was significantly increased in babies with positive findings of MC_{3-18\text{ m}} (51). In a study, where teenagers with ARC to pollen were challenge with pollen allergens outside the pollen season, we found a redistribution of MC towards the mucosal surface (52). This finding is in agreement with results from other studies (53, 54). An influx of basophils from the blood into the lamina propria and the epithelium in response to allergen challenge has been described (55, 56).

In order to analyse the possible influence on MC in non-allergic reactions, a study of nasal MC numbers and exposure to a reactive chemical was performed in a small group of healthy volunteers (57). The reactive chemical was dimethylbenzylamine (DMBA), which is used in epoxy systems in the production of plastic goods, and exposure to this compound is known to cause respiratory symptoms in epoxy workers (58). An increase of the nasal MC numbers
after DMBA exposure was found in a dose-response manner (57), which might indicate a role of MC also in non-allergic respiratory symptoms.

Nitric oxide and nasal nitrites/nitrates
In Paper III nNO and eNO levels were analysed in order to evaluate the ability of these markers, in comparison to acoustic rhinometry and spirometry, to differentiate between allergic subgroups with or without current allergen exposure and healthy controls. The correlation between nNO levels and nitrate/nitrite concentrations was also analysed.

In the respiratory tract the main production of nitric oxide (NO) comes from the upper airways and, above all, from the paranasal sinuses, where it is involved in different inflammatory diseases (59 - 60). In allergic inflammation, studies of nNO have given controversial results with findings of increased levels in allergic rhinitis patients as compared to controls in some studies (61 - 65) but no level differences in other studies (66 - 71). In contrast, eNO has been found to be a very useful marker in asthma diagnosis and in monitoring of asthma treatment (72 - 74).

An alternative method of studying NO production from the nasal cavity is analysis of the nitrites and nitrates in the nasal secretion, which are stable end products in the NO metabolism (75). Only a few studies have been performed using these markers in allergic rhinitis (76, 77), and no correlation between nNO and nitrite/nitrate levels was found in a study of paper-mill workers suffering from rhinitis (78).

A third method of studying nasal NO production is analysis of the different isoforms of NO synthases. These enzymes, involved in the NO synthesis from L-arginine and oxygen, have been studied in details in the mucosa of sinuses and the nasal cavity (79, 80). Higher amounts of NO synthases have been found in nasal mucosa from patients with allergic rhinitis compared to controls (81 - 85).

Gaseous nNO concentrations depend on which technique is used, by aspiration of nasal air or by single-breath nasal exhalation (86), as well as on simultaneous humming, which increases NO output from the paranasal sinuses (87). Furthermore, in evaluation of eNO levels, the salivary contribution of nitrates reflecting dietary intake of nitrate-rich food must be considered (88, 89). This NO contribution, which does not reflect airway inflammation, may be reduced by an antibacterial mouthwash (88). On the contrary, the nNO levels are not affected by nitrate intake or mouthwash, neither in nasally aspirated air (88), nor in nasally exhaled air (44).
**Markers with evaluations in progress**

*Palate lung nasal epithelial clone (PLUNC)*

PLUNC is a protein discovered a few years ago in nasal lavages and described as a potential marker of airway irritation (90). Decreased levels are found in smokers and in workers exposed to a reactive chemical (91). In a small group of subjects with ARC due to birch pollen the PLUNC levels were significantly lower compared to controls during the pollen season but not out of the season (92). The function of PLUNC is unknown, although it is suggested to be of importance in the innate immune defence of the upper airways. Further studies are of interest in subjects with allergic as well as non-allergic rhinitis in order to get information of the function of PLUNC and to evaluate the ability of this protein to differ between rhinitis groups.

*Clara cell protein 16 (CC16)*

Clara cell protein, CC16 (identical to CC10 and uteroglobin), is secreted by Clara cells, which are described as non-ciliated cells in the tracheobronchial tree. CC16 is proposed to serve as a sensitive marker of respiratory epithelial injury, as the Clara cells are particularly vulnerable to a number of air pollutants (93). Decreased levels of CC16 in serum and bronchial lavage have been found in relation to chronic lung damage with destruction of the Clara cells, caused e.g. by silicia and tobacco smoke exposure (94), while increased levels have been found after acute lung injury as e.g. exposure to ozone, explained by increased epithelial permeability with leakage of CC16 into the serum (95). Studies of Clara cells and CC16 in relation to asthma have shown decreased numbers of the cells (96), and decreased levels of the protein in bronchial lavages (97) in asthmatic patients compared to non-smoking controls. CC16 possesses immunomodulatory and anti-inflammatory properties (96), why reduced levels might contribute to airway inflammation and allergy (98, 99).

In the upper airways CC16 has been identified in nasal lavages (100). Lower concentrations were found in patients with birch-pollen induced ARC (101), and also on the gene level CC16 and other anti-inflammatory mediators were down-regulated in ARC patients in comparison to healthy controls (102). The CC16 levels were decreased after exposure to the reactive chemical DMBA (103). One of the anti-inflammatory properties ascribed to CC16 is the inhibitory effects on PLA₂. In a study of patients with pollen ARC, in which PLA₂ types were analysed by mRNA expression, the PLA₂ type VIIA was found in significantly lower amounts both during and out of season. This PLA₂ type down-regulates the platelet-activating factor
(PAF) (104), and decreased down-regulation of this inflammatory mediator might contribute to
the explanation of increased inflammation in allergic patients.
Thus, CC16 might be a useful marker in the differentiation between rhinitis groups, but needs
further evaluations.
RESULTS

Paper I

Allergy development

The development of allergy was achieved from all of the participants (n = 67) in the 6-years follow-up. Allergic symptoms were reported in altogether 29 children from skin and/or the respiratory tract, five children were regarded as probable atopic and 33 non-atopic. Since the first follow-up at 18 months of age (38), allergy symptoms persisted in 22/30 children and 19/25 remained healthy, while six children had recovered and seven had become allergic (Figure 1). Atopic eczema was the most common diagnosis (n = 19) followed by rhinoconjunctivitis (n = 16), asthma (n = 11) and urticaria (n = 5).

Metachromatic cell findings at 6 years of age

MC6 years were found in 40 of 54 cytospin slides evaluable for MC findings. The proportion of subjects with positive MC6 years findings was not different in the groups of atopy (19/24), probable atopy (4/5) and non-atopy (17/25). In the atopic group, nasal MC6 years were found in children with ARC with/without bronchial problems (11/13), in AB without nasal problems (2/3) as well in those with eczema without respiratory symptoms (6/8).

Metachromatic cells findings during infancy as predictors of allergy up to 6 years of age

The numbers of atopic children with demonstrable MC3-12 m (18/31; 58 %) were higher (p < 0.05) compared to atopic children without demonstrable MC3-12 m (10/33; 30 %). The predictive value of MC during infancy was regarded as low and not useful in clinical practice.

Paper II

Allergy development

The history of allergy symptoms was obtained from all of the 67 individuals in the third follow-up at 18 years of age. Allergy symptoms were reported by 39 individuals, probable allergy by four and no allergy by 24 individuals (Figure 1). There was a trend to a higher number of individuals reporting allergy symptoms among those attending the clinic for testing (31/49) compared to those who refrained from the visit (8/18). Since the previous 6-years follow-up, ten individuals reported debut of symptoms, two of the six individuals with previous recovery had regained allergy symptoms, and only one subject had recovered at this follow-up.
The most common diagnosis at this age was allergic rhinoconjunctivitis, reported by 33 of 39 individuals (85 %), and followed by eczema in 17 individuals (44 %) and allergic bronchial symptoms in 15 individuals (38 %). Non-allergic bronchial symptoms were reported by eight subjects; all of them, but one, were also suffering from other allergic manifestations.

**Prick test results**

Among the 49 individuals attending the clinic, sensitization was found to at least one allergen in all but one of the 27 individuals with respiratory allergy; thus, one male with a convincing history respiratory allergy to birch pollen was prick test negative. The prick tests were negative in all of the four individuals with symptoms limited to the skin, in three of the four individuals with probable airway allergy and in all of the 14 non-allergic individuals.

**Exercise test results**

The exercise test was performed in 47 individuals, as two individuals refrained from the test. Out of 11 individuals reporting allergic symptoms in the lower airways, the criteria of asthma diagnosis were fulfilled in only two of them, both suffering from perennial and pollen allergy, and nine were thus regarded to suffer from allergic bronchitis.
Metachromatic cell findings at 18 years of age

MC_{18 \text{ years}} were evaluated in 45 of the 49 individuals (Table 1), and the numbers of subjects with or without demonstrable MC in relation to allergic manifestations are shown in Table 2. The numbers of individuals with demonstrable MC_{18 \text{ years}} (16/30) were significantly higher (p = 0.01) in the group of individuals with allergy symptoms compared to the group of individuals with no allergy (1/12).

The proportion of subjects with positive MC_{18 \text{ years}} findings were not different in the subgroups of airway allergy with (9/17) and without (6/9) current allergen exposure, but the proportion was significantly higher in both of these subgroups (p < 0.02) compared to the healthy group (1/12).

<table>
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<tr>
<th>Allergy manifestations</th>
<th>MC positive</th>
<th>MC negative</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Airways +/- skin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- only pollens</td>
<td>6</td>
<td>3</td>
<td>26</td>
</tr>
<tr>
<td>- perennial +/- pollen</td>
<td>9</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Probable upper airways</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>No allergy</td>
<td>1</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Totals</td>
<td>19</td>
<td>26</td>
<td>45</td>
</tr>
</tbody>
</table>

Table 2. Number of subjects with and without demonstrable MC in relation to allergy manifestation.

Metachromatic cells findings during infancy and the family history of allergy as predictors of allergy up to 18 years of age

Nasal MC_{3-18 \text{ m}} were assessed in 64 infants (38) with positive findings in 33 and negative findings in 31 of them. During the period from birth up to 18 years of age, altogether 46 individuals were suffering (n = 38) or had been suffering previously (n = 8) from allergy symptoms (Figure1).
A significantly higher number of individuals (p < 0.001) developed allergy in the group with demonstrable MC 3-18 m (31/33; 94 %) compared to the group without demonstrable MC 3-18 m (15/31; 48 %). The occurrence of MC 3-18 m preceded the debut of allergy by many years in one third (11/33) of the individuals. All of the non-allergic individuals (n = 13) were MC 3-18 m negative. (Table 3).

Although the MC 3-18 m were demonstrated in the respiratory mucosa, symptoms remained limited to the skin in four of the individuals.

<table>
<thead>
<tr>
<th>MC during the first 3–18 months of life</th>
<th>First report of allergy at the follow-up</th>
<th>Probable allergy</th>
<th>Never allergy</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18 months 6 years 18 years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>present</td>
<td>20 5 6</td>
<td>2</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>not present</td>
<td>10 2 3</td>
<td>3</td>
<td>13</td>
<td>31</td>
</tr>
<tr>
<td>Totals</td>
<td>30 7 9</td>
<td>5</td>
<td>13</td>
<td>64</td>
</tr>
</tbody>
</table>

Table 3. Numbers of subjects reporting debut of allergy in relation to the nasal MC findings during infancy.

When the predictive value of FH was evaluated, development of allergy was found in 37 of the 44 individuals with a positive FH (84 %), and in 3 of 9 individuals negative FH.

A positive FH and/or demonstrable MC 3-18 m was found in 43/46 (93 %). However, the combination of a negative FH and no demonstrable MC (n = 8) did not exclude the risk, as two of them developed allergy problems.
Paper III

*Nitric oxide from the upper and the lower airways) and nitrites/nitrates in nasal lavage (NAL)*

The mean values of nNO were not different between any of the analyzed subgroups. In contrast, the mean value of eNO was significantly higher in the subgroup sensitized to perennial allergens compared to the other subgroups (Figure 2);

A slight reduction of both nNO and eNO after exercise was found in all of the subgroups, but reach significance only in the non-allergic group.

![Figure 2](image)

The mean values of nNO and eNO were only slightly lower in smokers compared to non-smokers.

Gender had an influence on the NO levels, as there was a significantly higher mean value of nNO levels in females compared to males, but the reverse of values between gender with lower mean value of eNO in females compared to males.

There were no statistical differences of the nitrite/nitrate levels between any of the allergy subgroups and the healthy group, and no correlations were found to gender or smoking habits.

There was a weak positive correlation between nNO and nitrite/nitrates in males before ($r^2 = 0.44; p = 0.03; n = 15$), but not in females.
Acoustic rhinometry and spirometry

No statistical differences in the mean values of VOL2 were found between any of the subgroups before or after exercise. No significant differences were found between any of the subgroups in any of the repeated FEV1 measurements.

Smoking habits affected the VOL2 values. Before the exercise the mean value of VOL2 was slightly lower in non-smokers compared to smokers (6.39 ± 1.12 cm³; n = 37 vs. 7.35 ± 1.69 cm³; n = 7). After exercise VOL2 increased significantly in non-smokers (p < 0.0001) while the values remained unchanged in smokers, resulting in a significant difference between smokers and non-smokers (8.80 ± 1.59 cm³ vs. 7.34 ± 1.11 cm³; p = 0.02).

The FEV1 values were significantly lower in smokers compared to non-smokers (90.0 ± 7.2 % vs. 98.7 ± 8.1 %; p < 0.01), and this difference was found in all of the repeated FEV1 measurements.

A positive correlation between eNO and Eos numbers in the peripheral blood was found in the subjects sensitized to perennial and pollen allergens (r² = 0.6; p = 0.05; n = 24), but not in the non-sensitized group.
GENERAL DISCUSSION

Methodological aspects

Longitudinal cohort studies are needed in the evaluation of early biomarkers of allergic development in children. However, when searching for markers, differentiating between allergy subgroups, there is a risk that the cohort will end up in several groups of subjects, which might be too small for statistical analyses.

In all studies it is of great importance to include a relevant control group. In this cohort study, the initial aim was to include a control group of one third of the study population remaining healthy, why families reporting allergy diseases and no problems were invited in a predetermined proportion. The detailed examinations of families already included, resulted in a group of babies with a possible family history, reducing the numbers of babies remaining as healthy controls with no heredity to less than 10 % of the study population. This demonstrates the importance of a very detailed interview to get correct information of the family history.

The diagnoses in the follow-ups were based on a detailed history of allergy symptoms, without including specific allergy tests. In this way all of the subjects, including those who did not attend the clinic, could be evaluated on the same base concerning allergy development. Actually, the information from the histories of symptoms were in a very good agreement with the prick test results (Paper II), why the subjects evaluated at a distance by questionnaires only or telephone interviews were reasonably right classified into the different groups of allergy manifestations.

The follow-ups at 6- and 18 years were performed during the non-pollen season in order to have the same environmental condition to all of the tested participants. This resulted in subgroups of subjects with respiratory allergy with and without current allergen exposures, and as a consequence presumably different concentrations of nasal inflammatory markers. One of the aims at the 18-years follow-up was to find nasal markers with ability to differentiate between allergy groups with or without current of allergen exposure period as well as healthy controls. This situation corresponds to clinical practice with allergy investigations in patients mostly performed out of pollen periods.

The method of MC analysis used in the follow-ups was the only available method at the start of the cohort study. The staining procedure does not differentiate between mast cell subtypes
and basophils. The numbers of MC, if occurring, is definitely in a minority as compared to the epithelial cell numbers, why there is a risk of over-looking single MC, resulting in false negative results. Thus, if absence of MC findings are true or false negative findings, can only be ruled out by using a more sensitive method for cell analysis. The method of laser scanning cytometry (LSC), described by Voltmann et al (105), using laser and specific antibodies for each MC subtype would probably identify even low numbers of specific cells, avoiding false negative MC findings, and also give interesting information by differentiation between MC subtypes.

Because the analysis of exact numbers of MC in relation to the total amounts of cell materials on the slides is not fully reliable, the quantification of MC numbers has not been used in the 18-years follow-up, where the result are reported as positive or negative cell findings.

Aspects on the study results
In this cohort study, the development of atopic manifestations from birth to adolescence followed the "atopic march", with eczema as the most common problem during the first years of life, gradually changing to respiratory allergy with ARC as the most common problem in young adult age. Only a few individuals had a lasting recovery from their allergy (5/35, Figure 1).

The original aim of the study was to evaluate the occurrence of nasal MC during infancy as predictors of allergy development. Although all of the infants with positive cell findings developed allergy or probable allergy (in a few subjects), negative cell findings did not exclude this risk. Thus, early-in life occurrence of MC, visualized with the method used in these studies, can not be used as a single predictor. However, the predictive value of FH might be improved if positive MC findings are added.

Detection of the MC3-18m was found to precede or coincided with positive skin prick test in a majority of babies as well as to precede the debut of allergy symptoms in a majority of children at the first follow-up at 18 months of age (38) and by many years with symptom debut not until adolescence in some subjects (Paper II).

These findings have caused us to speculate, that subjects with a propensity of allergy development might have a constitution with a higher MC numbers, and the presence of MC might have been able to contribute to the sensitization according to findings in studies on the role of mast cells as promotors of local IgE synthesis via B cell activation (106, 107). The
mast cell has been suggested to be a crucial effector cell in the pathogenesis of asthma, especially asthma with an allergic basis, as mast cells have been reported to be increased in numbers within the airway smooth muscle bundles of asthmatic patients (108). We can only speculate that many of the MC3-18m are mast cells, and that there is a corresponding occurrence of the same cell types in the bronchial wall, thus possibly contributing to asthma development. It is also interesting to note that detected MC 3-18m in nasal mucosa were found, not only in individuals developing respiratory allergy, but also in individuals with dermatitis without airway symptoms, which is in agreement with the concept of atopy as a systemic disease.

The additional aims to find nasal markers differentiating between allergic inflammation in different phases of allergen exposure and a normal mucosa were analysed in the 18 years follow-up (Paper II, Paper III). The results are summarized in Table 4.

<table>
<thead>
<tr>
<th>Marker/test</th>
<th>Airway allergy exposure</th>
<th>Atopic eczema</th>
<th>Probable allergy</th>
<th>No allergy</th>
<th>Group differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>(grouping according to Paper III)</td>
<td>I</td>
<td>II</td>
<td>III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCpositive / whole group (number of subjects; n = 45)</td>
<td>9/17</td>
<td>6/9</td>
<td>1/4</td>
<td>2/3</td>
<td>I/III p=0.02 II/III p=0.02</td>
</tr>
<tr>
<td>nNO (mean value, ppb; n = 40)</td>
<td>69 ± 31</td>
<td>62 ± 29</td>
<td>79 ± 20</td>
<td>66 ± 14</td>
<td>75 ± 28 n.s.</td>
</tr>
<tr>
<td>Nitrites/nitrates (mean values, µM; n= 37)</td>
<td>16 ± 7</td>
<td>18 ± 11</td>
<td>13 ± 3</td>
<td>13 ± 1</td>
<td>16 ± 9 n.s.</td>
</tr>
<tr>
<td>eNO (mean values, ppb; n=43)</td>
<td>47 ± 52</td>
<td>14 ±10</td>
<td>8 ± 2</td>
<td>9 ± 4</td>
<td>13 ± 5 I/III p=0.03 II/III p=0.06</td>
</tr>
<tr>
<td>Acoustic rhinometry (mean values, VOL2, cm3; n=40)</td>
<td>6.1 ± 1.4</td>
<td>6.4 ± 1.2</td>
<td>7.3 ± 0.2</td>
<td>6.1 ± 1.3</td>
<td>6.8 ± 1.4 n.s.</td>
</tr>
<tr>
<td>Spirometry (FEV1, %; n=45)</td>
<td>97 ± 9</td>
<td>98 ± 0</td>
<td>99 ± 10</td>
<td>97 ± 10</td>
<td>96 ± 8 n.s.</td>
</tr>
</tbody>
</table>

Table 4. Summary of marker and test results in Paper II and Paper III.
A significantly higher number of individuals with respiratory allergy had, as expected, demonstrable MC compared to healthy subjects, but the proportion of subjects with MC positive findings were not different in the groups with or without current allergen exposure. Thus, MC findings can not serve as a marker with ability to differentiate between the allergic subgroups, and due to too many negative findings among the allergic subjects MC is not useful to differentiate between allergic and groups either.

In this study no differences of nNO levels were found between of the groups and healthy controls. Neither were any differences of nitrite/nitrate concentrations found between any of the groups. However, the eNO was significantly higher in the allergic group with current exposure, which is agreement with NO as a well-known marker of inflammation in the lower airways.

The difference in eNO due to gender with lower values in females was in agreement with previous studies (44), but the reverse difference of nNO with higher values in females was unexpected. A possible explanation that NO production may be related to the menstrual cycle (109) needs further studies.

The functional tests showed no different results in the allergy and healthy groups neither by acoustic rhinometry in upper airways, nor in the lower by spirometry. This was possibly due to the tests being performed in a period with subjects in their best condition outside the pollen season. If the tests had been performed during the peak of pollen seasons, the results might have verified asthma in the subjects reporting pollen related allergic bronchial symptoms.

An unexpected finding were significant differences in both rhinometric and spirometric results in relation to smoking habits, in contrast to NO findings with only slight and non-significant reductions in smokers compared to non-smokers. Smoke related impaired lung function in adolescents with regular daily consumption is described in a large cohort study (110). The findings in our small groups are worth observation, as the smoking habits with relative low consumption and of relatively short duration in these young subjects caused significant reductions of FEV\textsubscript{1} values, although the results still were within normal limits.
CONCLUSIONS

- Detectable nasal MC during infancy were found to predict allergy development, and the cell findings often preceded the allergic symptoms.

- Nasal MC during infancy can not be used as a single biomarker for prediction of allergy development, due to absence of MC findings in a high proportion of subjects with developing allergy, but positive MC findings in combination with the family history of allergy can improve the risk evaluation.

- Differences between groups with and without current allergen exposure and the healthy group was detected by:
  - exhaled NO
  but was not found by means of:
  - nasal MC
  - nasal NO
  - nitrites/nitrates levels in nasal lavage
  - acoustic rhinometry
  - spirometry

- There is a need to find nasal markers, which can improve the differentiation between allergy, other rhinitis groups and healthy controls. Materials are achieved at the 18-years follow-up, and are under investigations of:
  - PLUNC concentrations in NAL and identification of PLUNC producings cells;
  - CC16 concentrations in NAL and nasal Clara Cells.
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