# 1. General information

## 1.1 Coordinating investigator 1

<table>
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<tr>
<th>Role</th>
<th>Contact</th>
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<tr>
<td>Dr. R. Lutter</td>
<td>Academic Medical Centre</td>
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<tr>
<th>Institute</th>
<th>Respiratory Medicine</th>
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<tr>
<td>Postal address</td>
<td>Meibergdreef 9, room K0-150</td>
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<tr>
<td>Zipcode + city</td>
<td>1105 AZ Amsterdam</td>
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<tr>
<td>Telephone</td>
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<td>020-5669001</td>
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<td><a href="mailto:r.lutter@amc.uva.nl">r.lutter@amc.uva.nl</a></td>
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## 1.2 Financial administr.

<table>
<thead>
<tr>
<th>Role</th>
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<tr>
<td>K.M. de Vries</td>
<td>AMC Medical Research B.V.</td>
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<tr>
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<th>Contract manager</th>
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<tr>
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1.3 Title of project:

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<tr>
<th>Language</th>
<th>Description</th>
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<tr>
<td>English</td>
<td>Targeted therapy for virus-induced exacerbations in asthma</td>
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<tr>
<td>Dutch</td>
<td>Gerichte behandeling van virus-geïnduceerde astma exacerbaties</td>
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Project number 3-2-10-069

1.4 Time schedule:

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<td>Start of project</td>
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<tr>
<td>Duration of project</td>
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<tr>
<td>Period of funding</td>
<td>from 01.11.2010 (dd-mm-yyyy) till 01.11.2013 (dd-mm-yyyy)</td>
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<td>Due to pregnancy leave of the post-doc on this project, Dr. S.M. Bal, the duration of the project was extended for 4 months, to 01.03.2014</td>
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1.5 Grant  € 149,354

1.6 Short description of the project for public information (in Dutch, see guidelines) (max. 250 words):

**Achtergrond**
Virus-geïnduceerde exacerbaties en overdreven ontstekingsreacties die daarmee gepaard gaan behoren tot de belangrijkste complicaties van astma, maar de onderliggende mechanismen die tot deze exacerbaties leiden zijn niet bekend.

**Belangrijkste bevindingen**
Eosinofiele granulocyten die als boosdoeners in het ontstekingsproces bij astma bekend staan blijken in staat om virussen te binden, op te nemen en af te breken. Deze beschermende functie van eosinofielen was nog niet bekend. Als gevolg van virusopname door eosinofielen worden deze cellen licht geactiveerd, hetgeen voldoende is om ze ontstekingsbevorderende componenten te laten maken. Voorlopige resultaten wijzen er op dat eosinofielen door blootstelling aan virus ook langer overleven en daardoor mogelijk meer schade kunnen berokkenen. Een van de bronnen voor schade zijn reactieve zuurstofprodukten (ROS). Omdat uit parallelle studies bleek dat een beperkte capaciteit van cellen om ROS schade te beperken correleerde met de ernst van de exacerbatie is dit ook geanalyseerd. De resultaten tonen dat zelfs bij blootstelling aan een lage dosis virus, cellen in de long niet meer goed kunnen reageren op ROS en meer ontstekingsbevorderende componenten gaan maken. De inclusie voor de klinische studie is vertraagd maar wordt de komende maanden voltooid waarna bekend zal worden of het blokkeren van IL-5 de schade door eosinofielen en door ROS beperkt wordt.

**Vooruitzicht**
Neutralisatie van interleukine-5 zorgt ervoor dat patiënten met eosinofiel astma langer gevrijwaard zijn van exacerbaties en minder afhankelijk van corticosteroïden zijn. Onze bevindingen suggereren dat beperken van overmatige activatie en van een langere levensduur van eosinofielen een betere astma controle geeft.

2. Report

2.1 Summary:
Title | Targeted therapy for virus-induced exacerbations in asthma
---|---
Authors | Dr. R. Lutter, Prof. dr. P.J. Sterk, Dr. K. van der Sluijs
Dept./Institute(s) | Departments of Respiratory Medicine and Experimental Immunology
Keywords (max. 6) | Asthma, exacerbation, virus, interleukin-5, mepolizumab

Abstract (max. 250 words):

Exacerbations of asthma are predominantly caused by respiratory viral infections, but the underlying mechanisms have not yet been elucidated. As anti-IL-5 (mepolizumab) has been shown to reduce exacerbation rates in asthma, we hypothesized that IL-5 impairs antiviral responses in asthma patients. This was addressed in a double-blind placebo-controlled trial with mepolizumab in mild to moderate asthma patients, who were challenged with rhinovirus16 (RV16), and by exacerbation studies in murine models and in vitro approaches. We found that eosinophils promoted clearance of respiratory viruses in murine models. Within 2h after eosinophils were exposed to respiratory virus, eosinophils took up and degraded virus. This was shown for human peripheral blood and bronchoalveolar lavage fluid eosinophils, from mice and asthma patients, and for RSV, influenza and rhinovirus. As a consequence of viral uptake, eosinophils were mildly activated (enhanced CD69 expression) and released pro-inflammatory mediators (IL-8 and IL-6), but no release of granular eosinophil cationic protein. In RV16-challenged patients, the CD69 expression on eosinophils correlated significantly with the asthma control questionnaire score, which indicates that this mild activation of eosinophils is clinically relevant. Preliminary studies also indicate that apoptosis of these mild activated eosinophils was delayed, which together with IL-5 could explain the enhanced and prolonged eosinophil response to a viral challenge in asthma. The clinical study will be closed soon, after which we expect to be able to determine whether IL-5 worsens eosinophil responses to a viral challenge. Specific interventions targeting activation and prolonged survival of eosinophils may dampen virus-induced exacerbations.

2.2 Description of original question/aim (max. 150 words):

**Problem**

Virus-induced exacerbations represent the major clinical manifestation of asthma, but underlying mechanisms are poorly understood. Neutralization of interleukin-5 reduces the exacerbation rate in mild to moderate asthmatics with eosinophilic inflammation.

**Hypothesis**

Since virus-induced asthma exacerbations are associated with enhanced Th2 and reduced Th1 (antiviral) responses, we hypothesized that interleukin-5 critically impairs the antiviral response in allergic asthma patients leading to:
- attenuation of cellular anti-viral responses
- prolonged/exaggerated inflammatory response
- enhanced asthma symptoms, airflow limitation and bronchial hyperresponsiveness

**Specific research questions**

Does IL-5 neutralization

1) reduce inflammatory response to viral challenge in allergic asthma?
2) reduce asthma symptoms to viral challenge in allergic asthma?
3) enhance cellular immune response to viral challenge in allergic asthma?
4) enhance virus-specific T cell responses *in vivo*?

**Approach**

The study comprised a double-blind placebo controlled study using humanized anti-IL-5
2.3 Results (max. 2500 words, please submit a maximum of 4 figures and diagrams separately):

**General study set up and phasing**

**Experimental mice studies:** Ethical approval was sought and acquired for influenza infection in HDM-sensitized mice and IL-5 transgenic mice. These studies were performed mainly during the first two years of the study.

**Clinical Study:** The contracting phase with GSK and the delivery of mepolizumab, inclusive of the GMP labeling of mepolizumab vials delayed the study by about a year. The clinical study started March 2012. Despite active recruitment, patients were hesitant to participate, particularly the repeated infusion with mepolizumab and therefore long duration of the study proved to be a hurdle. After seeing over 90 patients and with no patients included we have, in agreement with all parties, METC and CCMO, amended the study protocol so that only 1 infusion was required. Inclusion following the new protocol started January 2013. Since, and despite pregnancy leave of the leading researcher on the study, we have screened 71 patients, enrolled 32 patients of which 23 have completed the study and 2 are still within the study. Seven patients dropped out for various reasons, but not because of adverse events. Based upon the current recruitment pace we expect to have completed inclusion (28 patients) by the end of November 2014 and the last subject's last visit in February 2015. Analyses have been done for a number of parameters, and an analysis plan is being made for efficient analyses after the last subject's last visit.

**General:** As the first coordinating researcher accepted another position, coordination was taken over by René Lutter half way through the study.

**Experimental studies in mice**

We have employed two experimental models to study the role of IL-5 in virus-induced responses and used a third model for comparison.

For the **first** model, referred to as an acute HDM model, mice (Balb/c) were intranasally sensitized and challenged with house dust mite (HDM, Greer Laboratories, Lenoir, NC) as described by de Boer et al. (Lipopolysaccharide inhibits Th2 lung inflammation induced by house dust mite allergens in mice. Am J Respir Cell Mol Biol 2013;48:382-389). Upon sensitization and challenge with HDM the mice developed AHR to metacholine (Figure 1A) and significantly elevated levels of both total and HDM-specific IgE compared to saline treated animal (Figure 1C and D). Furthermore, HDM exposure elicited an eosinophilic inflammation in the lungs, as 40% of BAL cells were eosinophils (Figure 1B). This indicates that sensitization by this protocol leads to allergic symptoms in the mice. Mice were challenged with 10TCID50 A/PR/8/34 influenza. Controls were Balb/c wt mice.

The **second** model were IL-5 transgenic animals (NJ.1638) that constitutively express IL-5 under the CD3δ promotor in peripheral T cells (Lee et al., Expression of IL-5 alters bone metabolism and induces ossification of the spleen in transgenic mice. J. Clin. Invest. 2001 Apr;107(8):949-59). These mice have chronically elevated plasma IL-5 levels and eosinophilia in amongst others the general circulation, spleen, lung tissue and BAL. Controls were wild-type (C57BL/6) littersmates. Mice were exposed to 10TCID50 A/PR/8/34 influenza.

And for comparison, the **third** model (in collaboration with Dr. L. Ravanetti) is referred to as a chronic HDM model (Balb/c) in which mice were exposed on a daily base to HDM (25 microgram/exposure) for 5 weeks according to Fattouh et al. (Transforming growth factor-beta regulates house dust mite-induced allergic airway inflammation but not airway remodeling. Am J Respir Crit Care Med. 2008;177(6):593-603). The inflammation and tissue remodeling were persistent, but otherwise the pathophysiology resembled that in the acute model. These mice were challenged with 20TCID50 X31 influenza.

**The impact of a viral challenge in the three models**

A challenge with 10TCID50 A/PR/8/34 influenza in the chronic HDM model proved lethal, whereas these mice survived a challenge with 20TCID50 of the less virulent X31 strain of
influenza. Mice in the acute HDM model and IL-5 transgenic mice, however, coped well with the virulent A/PR/8/34 influenza. In fact, these mice did significantly better than their controls as is evident from a reduced loss of weight compared to their controls (Figure 2A and C). In contrast, the chronic HDM mice in comparison to their controls did worse in response to the viral challenge, despite the lower virulence of the used virus (Figure 2E). The viral load in both the acute HDM mice and IL-5 transgenic mice were cleared faster in comparison to their controls (Figure 2B and D). For the IL-5 transgenic mice a faster clearance was manifest despite higher viral loads at day 4 after exposure.

The airway hyperresponsiveness (AHR) to metacholine after viral infection was not different between the acute HDM mice and IL-5 transgenic mice and their respective controls. In chronic HDM mice, however, the HDM-sensitized mice showed a significantly higher AHR than their non-sensitized counterparts.

**Cellular infiltrate upon viral infection**

Viral infection in the acute model elicited a similar increase in total leukocyte numbers in BAL of HDM-exposed and control animals. However, whereas in both groups at day 4 after infection this influx was predominantly neutrophilic, in the HDM-sensitized animals the viral infection also strongly augmented the number of eosinophils. These eosinophils persisted until day 8 after infection, when eosinophil numbers in HDM-sensitized mice that were challenged with PBS had already declined. At day 8 a lymphocyte influx was observed, which was comparable in both infected groups. In comparison to the controls, in the IL-5 transgenic model a similarly enhanced and prolonged eosinophilic response was found to the viral challenge. In contrast, in the chronic model there was only a short transient enhanced eosinophilic response, followed on by a significantly enhanced neutrophilic response.

**Inflammatory cytokines**

In lung homogenate from acute HDM mice at day 4 after infection the levels of IL-4, IL-5, IL-13 and IL-25 were in between those from mice exposed to HDM only and to influenza only. This also applied to IFN-γ but at day 8 after infection. Strikingly, there was a strong increase in IL-33 in the lungs of acute HDM mice after a viral infection. IL-33 has recently been identified as a stimulus for type 2 innate lymphoid cells that can promote eosinophilic responses, and also mast cell and basophil responses. In chronic HDM mice within the first 4 days after infection, besides IL-33, also TSLP, IL-25, IL-13, IL-1α and β, IFN-γ, IL-17A and KC were significantly enhanced. At day 6 after infection also IL-6, IP-10 and MCP-1 were significantly enhanced. Interestingly, the levels of amphiregulin in acute HDM mice exposed to influenza as compared to non-sensitized mice exposed to influenza were significantly higher at day 4 and significantly reduced at day 14. Amphiregulin has been implicated in epithelial repair and thus these findings suggest that epithelial repair and thus recovery from the viral challenge is faster in the acute HDM mice. This is in line with the observed earlier recovery from weight loss after viral infection.

**Answers to the specific questions (2.2) that were obtained with the murine studies?**

1). IL-5 enhances inflammatory response to viral challenge in allergic asthma?
   - IL-5 enhanced and prolonged eosinophil numbers and did not affect any other cell types. The amounts of pro-inflammatory mediators in the acute HDM model were less than in the chronic HDM mice.

2). IL-5 enhances (asthma) symptoms to a viral challenge (in allergic asthma)?
   - IL-5 reduces clinical symptoms triggered by a viral infection. The same is true for the acute HDM mice, but not for the chronic HDM mice, suggesting that in a chronic allergic setting the protective effect of IL-5 is overruled.

3). IL-5 reduces cellular immune response to a viral challenge in allergic asthma?
   - IL-5 enhanced and prolonged only the eosinophilic response to a viral challenge, but not that of lymphocytes nor any other cellular responses. The enhanced IL-33 response in the acute HDM mice and chronic HDM mice challenged with virus may enhance the eosinophilic response via ILC-2, basophils or mast cells.

4). IL-5 reduces virus-specific T cell responses *in vivo*?
Although the IFN-γ production in these acute HDM mice was less than those in the non-sensitized influenza-challenged mice, clearly this was sufficient to clear the viral challenge. In the chronic HDM mice the IFN-γ production was even higher than in the control mice.

Together these findings suggested that eosinophils display an important antiviral role, but that this role is aberrant in an allergic setting. There are no indications that the pulmonary lymphocyte responses are attenuated in an allergic setting.

**Anti-viral role for eosinophils?**

IL-5 transgenic mice were challenged with a fluorescently (DID)-labeled A/PR/8/34 influenza and these mice were sacrificed 4h after infection for the analysis of cells in the bronchoalveolar lavage fluid and lung draining lymph nodes by flow cytometry. Eosinophils appeared to have taken up virus and displayed activation markers (Figure 3). These studies led us to analyze the antiviral role in more detail and focus on human eosinophils. Human eosinophils were isolated form peripheral blood by negative selection and were incubated *in vitro* with DID-labeled influenza. Within 2h, virus was bound diffusely by all eosinophils and accumulated in clusters on the eosinophil’s cell surface and apparently was taken up (Figure 4A). Similar findings were obtained with DID-labeled RSV and for human eosinophils collected from bronchoalveolar lavage fluid. DID-labeled heat-inactivated RSV was also taken up by eosinophils, suggesting that eosinophils are not infected by RSV. To further study this eosinophils were exposed to RSV-GFP (provided by Dr. L. Bont, UMCU). Whereas epithelial NCI-H292 cells 24h after exposure to RSV-GFP were expressing GFP, eosinophils did not express GFP. So, this confirms that there is no infection of eosinophils by RSV. Next we have followed DID-labeled virus bound by eosinophils by confocal microscopy (Figure 4B). Triple staining and stacks clearly show that virus co-localizes with major basic protein on the eosinophil’s cell surface. Intracellular DID-labeled virus co-localizes with the lysosomal compartment, which is suggestive of its degradation.

Interestingly, the eosinophils that take up virus display an enhanced CD69 expression and release IL-8 and IL-6, but no eosinophil cationic protein (ECP) (Figure 4C and D). Together this indicates that uptake of virus provides a mild stimulus, which is strong enough to trigger the release of pro-inflammatory mediators, but not for degranulation. Recent findings indicate that this mild activation markedly delays apoptosis of eosinophils, which is in line with the prolonged presence of eosinophils in influenza-infected acute HDM and IL-5-transgenic mice.

**Clinical study**

In the first paragraph of section 2.3 we have explained why, despite recruiting three times as many patients as anticipated initially, the last patient out will by February 2015. We are eager to round off this study not in the least as more recently it has become evident that mepoluzimab has a major corticosteroid-sparing effect, reduced exacerbations, and improved control of asthma symptoms in severe asthma patients (*Mepolizumab Treatment in Patients with Severe Eosinophilic Asthma, Ortega HG et al.; Oral Glucocorticoid-Sparing Effect of Mepolizumab in Eosinophilic Asthma, Bel EH et al., N. Engl. J. Med. 2014 Sep 8. [Epub ahead of print]).

To follow up on the antiviral properties of eosinophils we have obtained eosinophils by bronchoalveolar lavage fluid from allergic asthma patients 7 days after RV16 challenge. Eosinophils were analysed for enhanced CD69 expression and for RV16 as described above for human neutrophils. We have not been able to detect RV16 (antibody kindly provided by Prof. Jim Gern) in these eosinophils, which may relate to the relative low dose of virus in the bronchial compartment. However, the eosinophils obtained displayed enhanced CD69 expression. Moreover, the CD69 expression correlated significantly with the asthma control questionnaire (ACQ) (Figure 4). These findings support our hypothesis that eosinophils take up virus *in vivo* and that their mild activation is clinically relevant.

Activated eosinophils produce reactive oxygen species (ROS), which may lead to oxidative
stress and contribute to inflammation. Anti-IL-5 may attenuate ROS production by eosinophils. Since there is no data available on oxidative stress in virus-induced asthma exacerbations, we collected alveolar macrophages from sputum by negative selection, before and after RV16 challenge. Analyses of oxidative stress-induced post-translational modifications of proteins (4-HNE and carbonylation) showed that 10TCID50 of RV16 caused a profound oxidative stress. Overnight recuperation of the alveolar macrophages and subsequent exposure to reactive oxygen species (the superoxide-generating xanthine-xanthine oxidase system) showed that alveolar macrophages after RV16 had lost their capacity to counter oxidative stress whereas the alveolar macrophages collected before RV16 exposure could easily deal with the oxidative stress. Interestingly, alveolar macrophages not able to counter superoxide, produced various pro-inflammatory mediators (IL-1β, IL-8, IL-6 and TNF-α). These findings indicate that control of oxidative stress may limit inflammation during an exacerbation.

**Conclusions from human studies?**

To study the effect of mepolizumab we have to await conclusion of the clinical study. The studies so far have led to some important new insights. First of all, human eosinophils have the capacity to take up and degrade viruses. Previous studies, in particular those by Helene Rosenberg c.s., have shown that eosinophils may release granular RNAse activity that by an as yet unexplained mechanism should lead to eradication of viruses. Our findings for uptake of viruses by eosinophils are indicative of a very different mode of action. Above all, virus-exposed eosinophils are mildly activated, which leads to the release of pro-inflammatory mediators. Furthermore, preliminary data indicate that these eosinophils may survive over a prolonged period, which contribute to the pathophysiology in virus-induced loss of asthma control. The correlation between CD69 expression by bronchoalveolar lavage fluid eosinophils and the ACQ provides support to this postulate. In this setting IL-5 may further aggravate eosinophil responses, which would explain the protective effect of mepolizumab in eosinophilic asthma. In view of this, we expect that our clinical study with mepolizumab may provide further clues as to the effect of IL-5 during virus-induced loss of asthma control.

A second important finding is that alveolar macrophages loose the capacity to control oxidative stress during a low-dose RV16 infection. It is likely that this will also affect other local cells such as epithelial cells and immune cells, although this remains to be proven. Given that alveolar macrophages are abundant local cells, our findings likely bear clinical relevance. We will assess the impact of mepolizumab on oxidative stress. Further these findings warrant further studies towards therapeutic interventions to enhance the local anti-oxidant capacity.

2.4 Did the study solve the original question? yes/no (explain) (max. 250 words):

Since the clinical study is still ongoing this question cannot be answered as yet. From the murine studies it is clear that IL-5 can promote clearance of respiratory viruses, at the expense of an enhanced and prolonged eosinophil response. We predict that in allergic asthma this enhanced and prolonged eosinophil response is facilitating viral clearance, but may also promote inflammation and asthma symptoms. If so, anti-IL-5 may dampen these effects by eosinophils. Although the antiviral IFN-γ response was less in acute HDM mice upon exposure to influenza, these mice adequately cleared the virus and therefore the Th1 response is assumed sufficient. In addition, in chronic HDM mice the IFN-γ response was higher than that in control mice. From these studies we consider it unlikely that IL-5 attenuates the antiviral responses by lymphocytes.

3 Papers (see instructions)

3.1 All publications (published or submitted peer-reviewed manuscripts):


3.2 All publications (not peer-reviewed like abstracts, newspapers, websites, etc.):

2012
- Oral presentation at EEACI Winterschool, Are, Sweden
- Oral presentation + poster at Nederlandse Longdagen, Utrecht, The Netherlands
- Poster ATS, San Francisco, USA
- Oral presentation at Cross Company Respiratory Symposium, Horsham, UK

2013
- Oral presentation at Keystone meeting ‘Pathogenic Processes in Asthma and COPD’, Santa Fe, NM, USA

2014
- Poster ERS Lung Science Conference, Estoril, Portugal
- Poster EEACI, Copenhagen, Denmark

4. Implementation (see instructions):

The current findings provide a rationale for the reduced exacerbation rate of asthma by anti-IL-5, but with no complete data set for the clinical study as yet its implementation remains speculative. Nevertheless, based on current findings there are a number of questions that need to be addressed.

1. Whereas eosinophils display a pronounced antiviral role, contradictory, reduction of the peripheral blood eosinophils proves clinically favorably. From our murine studies one could conclude that anti-IL-5 may promote a predominantly neutrophilic response to a viral challenge. Future studies should be aimed to clarify whether this is the case and whether this could affect disease progress.

2. Our data together with the recent study by Ortega et al. and Bel et al. on mepoluzimab in severe asthma warrants further studies on severe asthma along the lines presented here for mild to moderate asthma.

3. The mechanism by which eosinophils take up virus and the apparent coinciding survival signal are unknown. A better understanding of this may lead to new therapeutic options. In collaboration with Prof. Geijtenbeek, who has an extensive background in binding of viruses to cells, we will apply for grants to clarify this.

4. Interventions that may promote apoptosis or reduce mild activation of eosinophils should be tested. In fact, we are currently performing a preclinical study with a Chiesi compound on virus-induced activation of eosinophils.

1 and 2 are crucial studies and for that we will need to seek collaboration with pharma companies. There are already close contacts with Ortega (GSK) in place and we envisage that based upon the results from this study further studies will be initiated. Studies like this will take 3-5 years to complete. For 3 we will apply for grants shortly. It is anticipated that the underlying mechanisms can be revealed in 3 years. For 4, we are currently performing a preclinical study with a Chiesi compound on virus-induced activation of eosinophils.
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