Final Publishable Summary Report

Executive Summary

Chronic inflammatory diseases associated with allergy, including asthma and rhinitis, constitute a major and continuously growing public health concern for Europe. However, the causative factors and mechanisms converting a physiological inflammatory reaction to a chronic response causing allergic disease remain elusive. Viral infections, particularly those associated with human rhinoviruses (RV) are the most frequent triggers of acute asthma attacks. RV infections have more recently been associated with asthma initiation and there is evidence suggesting that such infections may also contribute to respiratory allergy persistence. PreDicta evaluated the hypothesis that repeated infections reprogram the immune system towards a persistent inflammatory pattern leading to respiratory allergies, following three interconnected workflows: models, mechanisms and translational output.

Looking into infectious agents present in young children with asthma, we have confirmed that RV is the most frequent microorganism in attacks; however, using sensitive methods we were also able to isolate RV in a high proportion of healthy children and children with asthma away from symptoms. It is possible that the interaction of RV with the immune system is not confined to asthma attacks. A variety of strains circulated around Europe. Viruses interacted with bacteria and higher levels of colonizing bacteria, especially Streptococcus and Moraxella, were found in children with a viral infection. When RV infected epithelial mucosa together with Staphylococcus, the epithelial integrity was affected, resulting in biofilm formation and bacterial invasion.

The respiratory epithelium plays a central role in defending against external factors such as viruses, initiating and coordinating the response, by sending signals to other elements of the immune system. The bronchial epithelium of patients with allergic asthma is not able to produce adequate amounts of antiviral interferons; this is also the case for their nasal epithelium. The response to the virus is dampened or shut down very early in the course of the infection, resulting in an overall 'lazy' and inadequate epithelial response. Among molecules that regulate the antiviral response, we have found that SOCS-1, a factor controlling interferon, is overexpressed in asthma, suggesting a possible target for intervention. We also found that cytokines produced by the epithelium, such as IL25 and IL33, are capable and necessary for driving allergic immune responses in the lung.

The immune response during an asthma attack was different if the trigger was a virus or an allergen. RV is able to enter and possibly replicate in monocytes and B-cells, activating them. The exposure of T-cells to viral and bacterial triggers was able to break established tolerance through myeloid dendritic cells.

Furthermore, we have established a new method to measure lipid factors that influence the resolution of inflammation. Administering some lipid mediators such as Protectin D1 and Resolvin D1, resulted in reduction of inflammation. This suggests that these substances can be promising as anti-inflammatory agents. In children, lipid mediators appear to be depleted in serum after an acute exacerbation and recover slowly.

Important translational outputs of PreDicta include a peptide chip able to identify and differentiate antibody responses to different RV subtypes. We have found that antibodies to RV are misdirected towards non-protective epitopes; the evolution of antibody responses to different RV subgroups is variable.

Finally, we were able to design antisense molecules – DNAZymes – able to cleave RV in-vitro. These will be further taken forward as possible candidates for anti-RV agents.

The PreDicta interdisciplinary Consortium with its strong track record, unique resources and strong translational focus, has produced new knowledge and technologies that can rapidly and effectively reach clinical care in respiratory allergies such as asthma and rhinitis.

Context

Chronic inflammatory diseases associated with allergy, including asthma and rhinitis, constitute a major and continuously growing public health concern for Europe. In some countries, one in three children suffers from these conditions, but what causes an inflammatory reaction to become chronic is still unknown. The European Academy of Allergy and Clinical Immunology (EAACI) warns that, with increasing trends, in the next decade half of the European population may suffer. Asthma and rhinitis very frequently coexist, especially in severe cases, and terms such as 'respiratory allergy', 'combined upper and lower airway disease', and more, have been proposed to describe this condition. Currently more than 30 million European citizens suffer from chronic asthma, among which 6 million have severe symptoms, and 1.5 million live in fear of dying from an asthmatic attack. Asthma costs €17.7bn per year to European healthcare authorities, and another €9.8bn per annum to the European economy due to productivity loss (European Respiratory Society White Book, 2003). In fact, due to strict definitions, it is likely that these figures are underestimations. The burden of allergic and non-allergic rhinitis/rhinosinusitis is even higher: more than 150-200 million Europeans experience this problem. Although the majority of patients with rhinitis can control their disease through medication, a considerable proportion (estimated at around 20%) suffer from severe chronic upper respiratory disease (SCUAD) that is not controlled with medication and has a profound impact on quality of life. These numbers are expected to grow further in the coming decades, establishing asthma and rhinitis as major epidemics of our times and requiring immediate action for the development of novel preventive and/or therapeutic strategies for their management.

Considerable effort has been put and significant advances have been achieved in the last decade in the description and understanding of asthma and rhinitis in Europe and worldwide. Nevertheless, these advances have not been translated into equivalent improvements in the quality of life of patients, or breakthroughs in respiratory allergy treatment. In attempting to understand the reasons for such discrepancy, a number of issues have to be taken into account: First, it is becoming increasingly clear that current classifications, and consequently attempts to model the disease(s) or include subjects in research trials, are imprecise. It is now recognized that allergic respiratory symptoms can be the result of diverse, but frequently overlapping, mechanisms. From another perspective, there exist different patterns of disease presentation, i.e. phenotypes, which should be individually looked into. Finally, and possibly most importantly, the natural history of the diseases can be highly variable. For a long time, studies of asthma and rhinitis/rhinosinusitis pathogenesis have mostly focused on the understanding of pathways leading to Th2-mediated allergic airway inflammation at the level of allergen sensitization and allergen exposure [1]. Such research has heavily relied on animal models, often very sophisticated and cleverly designed, which can replicate several, but not all, features of allergen sensitization and/or challenge including Th2 cell infiltration, eosinophilia, IgE production and airway hyper-responsiveness. Although animal models have been instrumental in understanding the immunological mechanisms controlling allergic airway inflammation and allergic inflammatory responses in general, this has not translated to new therapies, even for well phenotyped allergic asthmatics and despite considerable investment by the industry. Rhinitis patients have benefited even less from these approaches. This is now attributed to the complexity that underlies human respiratory allergy and asthma that is far from a simple Th2-mediated disease and consequently poorly represented by current animal models [2]. For example, neutrophilic inflammation is common in human asthma and correlates with asthma severity, whereas neutrophilic inflammation without eosinophils is seen in a substantial group of severe-corticosteroid dependent asthmatics. Moreover, eosinophilia and mast cell degranulation is seen in non-IgE mediated asthma, which is not associated either with atopy or allergic sensitization and there is no allergen trigger. Finally, human asthma

is usually triggered and/or exacerbated by a wide range of environmental factors beyond inhaled allergens including exposure to air pollutants, certain drugs, occupational chemicals, environmental tobacco smoke and most importantly respiratory infections. As these are features that have been largely ignored in the past, a significant gap of knowledge exists with respect to the mechanisms contributing to asthma pathogenesis beyond, or in synergy with allergic sensitization. This gap is even larger in rhinitis and rhinosinusitis, chronic inflammatory diseases of the upper respiratory track that share many pathophysiological features with asthma but have very little been investigated for possible effects of viral infections in hyperresponsiveness-mediated clinical symptomatology. Asthma, as other allergic diseases, most frequently appears in early childhood and may persist for a long period of time. However, the course of the disease can vary widely over time. Patterns of remission, relapse and new disease development at any age, suggest that the natural history of the disease may not be deterministic, i.e. 'decided' at an early stage by either a genetic pattern or an environmental exposure, but rather indeterministic, i.e. continuously developing in relation to ongoing non-predictable exposures. However, the time element has been difficult to include in most disease models and this aspect of disease persistence has not been adequately evaluated. During the last decades, it has become evident that viral infections, particularly those caused by human rhinoviruses (RV) are the most frequent triggers of acute exacerbations of asthma; in some cases viral agents have been detected in more than 90% of such events [3]. RVs are also responsible for the majority of mild rhinitis, i.e. common colds, therefore contributing further to symptomatology in respiratory allergic patients. More recently, using prospective study designs, RV infections have also emerged as major determinants for the development of persistent wheeze/asthma [4]. The extent of this association has been remarkable, with lower respiratory RV infections during the first three years of life increasing the risk of children to develop asthma by the age of six up to 40-fold, a much higher degree than allergen sensitization or respiratory syncytial virus infections, also common in children [5]. Nevertheless, the involvement of viral infections in asthma and/or rhinosinusitis persistence is far less clear. Viral infections may increase allergen sensitization in predisposed atopic individuals; atopy may in turn predispose individuals to more frequent and more severe viral infections. RV infection may also promote airway remodeling through the production of growth factors, and this is augmented in an atopic environment [6]. Furthermore, a disease exacerbation, which is highly correlated to an RV infection, may increase the chances for a subsequent infection and/or exacerbation. In a recently described mouse model, a single viral infection resulted, in addition to the acute effects typically described, in development of chronic inflammation [7]. However, mouse models of RV infection have only recently been described [8] and whether the above effect can be generalized is unknown.

Main ideas behind PreDicta

- Rising incidence of asthma and rhinitis in Europe with high socioeconomic burden
- Urgent need for novel preventive, diagnostic and therapeutic approaches
- Strong recent evidence associating rhinovirus infections with the origins, triggering and persistence of asthma
- Need for understanding the pathophysiological mechanisms linking infections to inflammation persistence in asthma and rhinitis
- Need to explore an indeterministic approach in the persistence of asthma/ respiratory allergies
- Gap between scientific discoveries and translation into clinical practise
- Consortium with translational focus, balanced clinical cohorts and experimental models, track record, unique resources and technologies

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2. Anderson, G.P., Endotyping asthma: new insights into key pathogenic mechanisms in a complex, heterogeneous disease. Lancet, 2008. 372(9643): p. 1107-19.
3. Sykes, A. and S.L. Johnston, Etiology of asthma exacerbations. J Allergy Clin Immunol, 2008. 122(4): p. 685-8.
4. Kusel, M.M., et al., Early-life respiratory viral infections, atopic sensitization, and risk of subsequent development of persistent asthma. J Allergy Clin Immunol, 2007. 119(5): p. 1105-10.

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6. Psarras, S., et al., Vascular endothelial growth factor-mediated induction of angiogenesis by human rhinoviruses. J Allergy Clin Immunol, 2006. 117(2): p. 291-7.
7. Kim, E.Y., et al., Persistent activation of an innate immune response translates respiratory viral infection into chronic lung disease. Nat Med, 2008. 14(6): p. 633-40.
8. Bartlett, N.W., et al., Mouse models of rhinovirus-induced disease and exacerbation of allergic airway inflammation. Nat Med, 2008. 14(2): p. 199-204.

Aims and hypothesis

PreDicta has set the aim of improving health and quality of life of individuals affected by respiratory allergies, by filling in critical gaps in our understanding of the pathogenesis of the disease and by suggesting new prevention programs and innovative treatment strategies.

The PreDicta central hypothesis was that repeated, acute infection-mediated events may reprogram the innate, adaptive and/or regulatory immune responses to predispose towards a chronic inflammation pattern.

Objectives and Strategy

The program was planned to look into the modulation of inflammatory patterns by acute infections in patient cohorts, mouse models and experimental in-vitro systems. The role of specific agents has been aimed in order to develop innovative diagnostics for predicting disease chronicity, as well as intervention strategies that may delay and/or prevent disease progression/persistence, by targeting the causative agents and/or specific elements of these inflammatory pathways. The overall objectives included the investigation of the potential lack of resolution as a cause for the chronic nature of the inflammation process; the identification of microbial agents, altered host-pathogen interactions, molecules and pathways that mediate establishment and persistence of chronic inflammation in allergic diseases and the use of this knowledge towards the development of new preventive, diagnostic and therapeutic strategies and products against respiratory allergies.

To advance the present knowledge on the role of infections in the persistence of a chronic inflammatory response, and deliver tangible products that can impact clinical care, PreDicta partners have agreed to focus on allergies of the respiratory tract, as a unique model system to achieve the following specific objectives:

- Associate asthma/rhinitis persistence with the number and/or type of respiratory infections including the role of emerging respiratory viruses, such as RV-C
- Develop mouse models of repeated RV infection, with or without allergen, and evaluate the resulting inflammation patterns
- Evaluate differences in primary epithelial cell responses to viral infection between atopic and non-atopic, asthmatic and normal individuals
- Analyze interactions between viral and bacterial agents in inflammation induction
- Study the effects of viral infection on T-cell mediated immune regulation
- Examine the role of specialized pro-resolution mediators in virus-induced inflammation
- Evaluate the possibility of predicting asthma persistence through virus exposure assessment
- Develop antiviral and/or anti-inflammatory DNAzymes aiming at preventing disease persistence

PreDicta followed three interconnected work-flows: models, mechanisms and translational output that were structured into several tasks within the project.

Main results and achievements

PreDicta has made remarkable progress towards understanding the mechanisms leading from infection to inflammation and hyperresponsiveness, as well as a big step towards bringing the mechanistic findings closer to bedside. Over 70 publications in high level journals have documented PreDicta findings so far, while the available datasets and biological material are expected to sustain PreDicta's legacy and will help support the next steps towards facing the allergy epidemic, while generating many more scientific impact through publications, conference talks etc.

We describe below a synthesis of the findings, starting from the viruses associated with respiratory allergy exacerbations and probably persistence, going on into cellular and molecular biology findings, followed by in-vivo data and finally translational outputs.

Viral agents associated with asthma in preschool children

Preschool children that took part in the 2-year follow up of the pediatric cohort were screened at baseline and during acute events for the presence of respiratory viruses. Although it is well established that acute asthma exacerbations are associated with respiratory viral infections, most often because of a RV, there are still many open questions in relation to the frequency, type and relevance of each virus. Furthermore, we wanted to associate these findings with the immunological responses monitored in these children.

We used both in-house and commercially available PCR methodologies with a high sensitivity and specificity in order to identify the viruses. In addition, we have sequenced all RV strains that were identified.

An intriguing finding of this study was that the majority of preschool children with asthma (60%), as well as healthy children (64%), harbored respiratory viruses at baseline, when they were well. RV was the agent most often identified, in around 70%-80% of cases. The asthmatic children had a tendency to harbour other respiratory viruses more commonly than healthy children. At the time of an exacerbation, the proportion of virus identification was of course higher in asthmatic children in comparison to the baseline (82%).

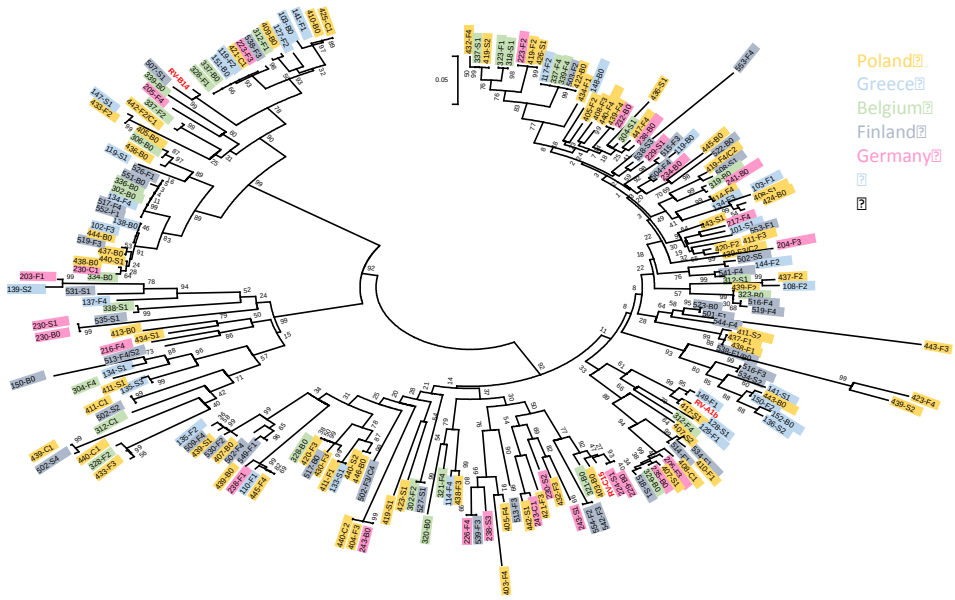

Figure 1 - Phylogenetic analysis of rhinovirus 5' non-coding region partial sequences from the viruses isolated in different countries

Genotyping was successful from 140 rhinovirus positive samples from asthmatics. Fifty-four strains represented rhinovirus A, 37 strains rhinovirus B and 49

strains rhinovirus C species. Interestingly, during symptoms mainly rhinoviruses from rhinovirus A and C species were found. In addition, RV C was relatively more frequent than RV A in asthmatic children than in controls. Phylogenetic analysis showed that different RV genotypes were simultaneously circulating in the participating countries during the study period (Fig. 1).

The immune response to different virus strains shares some basic characteristics, but quantitative differences are evident between the strains (Fig. 2)

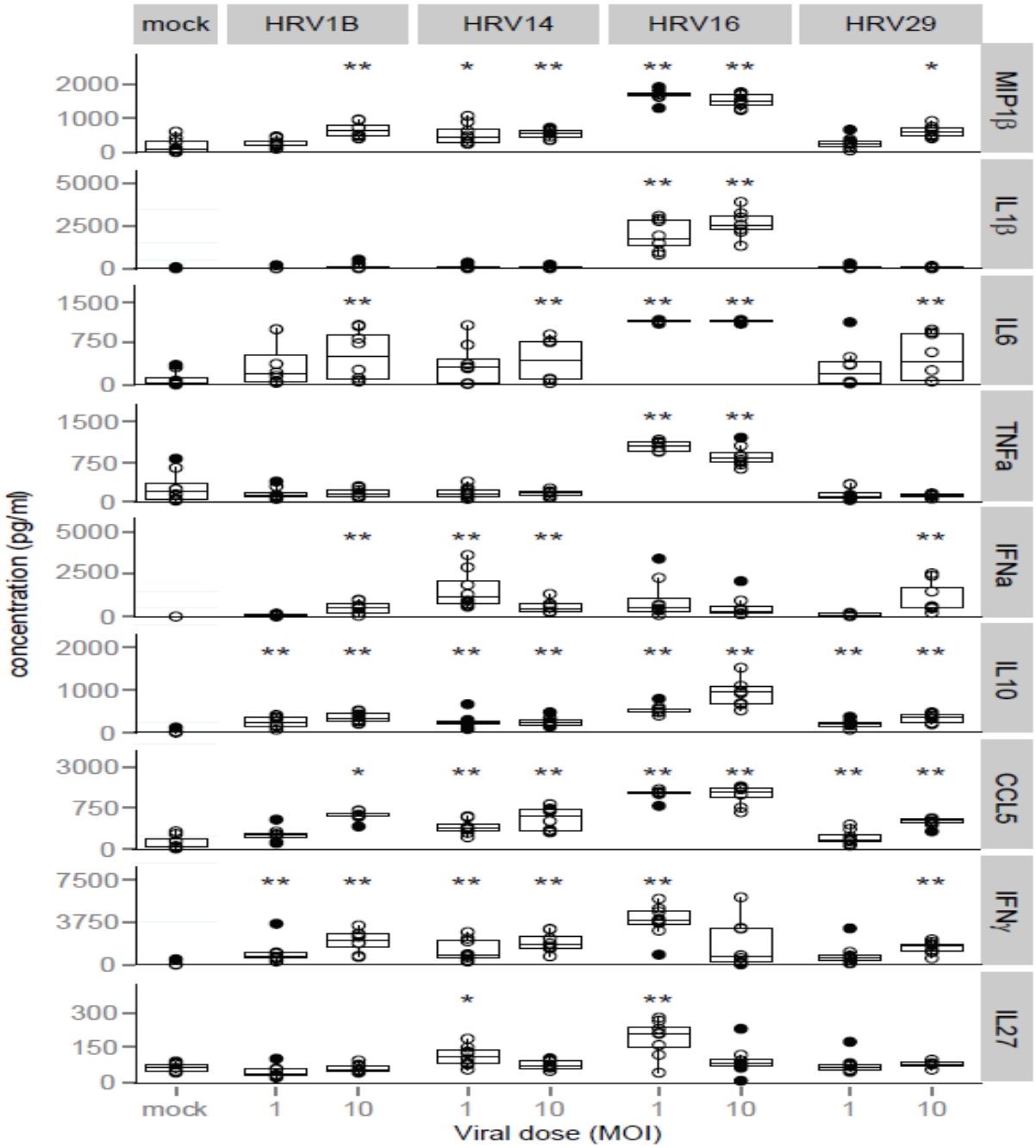

Figure 2 - RV-induced cytokine responses of PBMCs. PBMCs were incubated with HRV1B, HRV16, HRV14 and HRV29 at MOI = 1.0 and MOI = 10 concentrations or cultured in "mock" condition. Cell culture supernatants were collected at day 5.

Viral-bacterial interactions

While infection with either a viral or a bacterial pathogen may have a detrimental effect to the host, in reality many different microorganisms interact with the host in parallel; it is possible that these effects are combined or even synergistic. We have used an ex-vivo model of interactions between RV and Staphylococcus aureus (SA) infections in human nasal mucosa. We were interested to find out whether the combined infection may affect penetration and mucosal spread of one or the other microorganism and how it may affect the inflammatory responses. The model used inferior turbinate or nasal polyp tissue obtained from surgical material. Evaluation of RV16 and SA mucosal spread was performed by confocal microscopy. We found that epithelial cells were sloughed; however, the epithelial cell lining and borders remained structurally intact. We did not observe a breakdown of the basement membrane. RV as a single infection led to very few infected cells of the outer epithelial barrier within 48h; however RV may be able to enter more readily possibly due to its small size. SA as a single infection did not affect the epithelial integrity and only few bacteria attached to the epithelium. However, after infection with RV16 for 48h and SA for 24h the whole epithelial barrier was heavily infected by RV; SA was able to pass through the basement membrane and invade the mucosa (Fig.3).

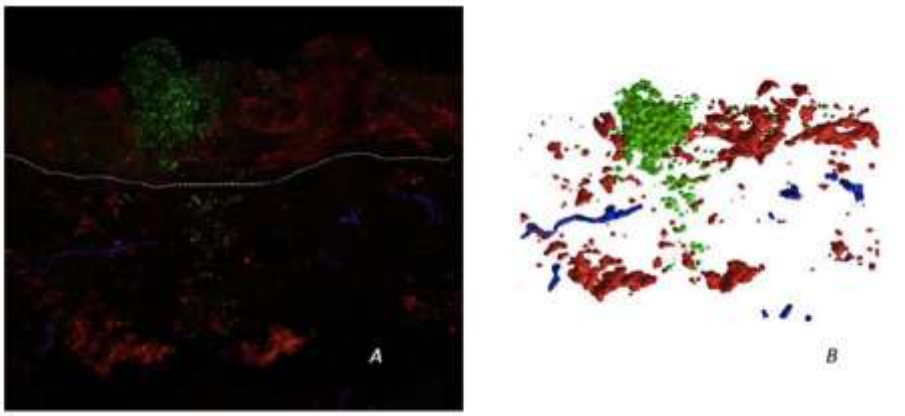

Figure 3: Inferior turbinate tissue of a normal control co- incubated with HRV16 and S. aureus for 24 hours. A: confocal microscopy, B: 3D reconstruction. HRV16 in red, S. aureus in green.

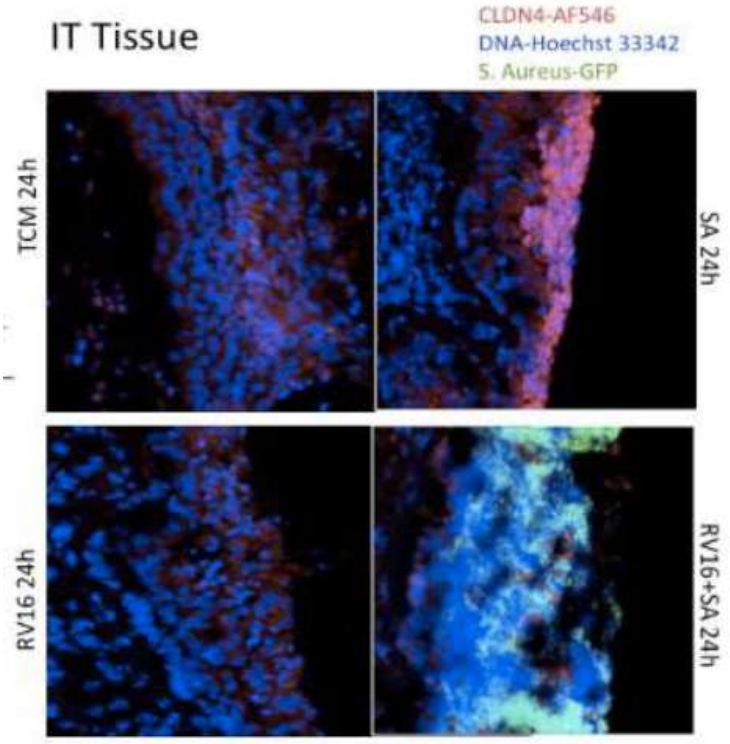

Figure 4 - SA (upper right) upregulates claudin-4. RV (bottom left) has little effect, however, the combination (bottom right) results in biofilm formation and invasion of SA into the tissue

This interaction involves molecules of the epithelial tight junction, such a claudin-4 and the specific tissue environment affects the response: important differences between control and polyp mucosa are observed. RV did not have much effect on epithelial tight junction molecules, in contrast to SA that was able to upregulate these molecules in normal, but not polyp, mucosa. When RV and SA was both present claudin was downregulated, the epithelial integrity affected resulting in biofilm formation and bacterial invasion (Fig.4).

The post-infection cytokine profiles of RV and SA were compared in control and polyp mucosa. IL-6 and IL-1b were clearly increased after 24 hours RV or SA infection. After RV infection, there is a deficit of IFNα and IFNγ release in NP vs IT, however, other IFNs were

not consistently measurable. There is also release of IL-17 and TNFa, however this shows clearly different patterns between IT and NP, specifically in IT, the release of IL-17 and IFNγ is increased whereas in NP, TNFa is released upon exposure to RV. The reason for this difference could be the cellular sources of these cytokines, with IL17 and IFNg mostly produced by T cells, and the major source of TNFa being macrophages and monocytes. The impaired IFN response, especially the IFN deficit in NP, could implicate that this nasal tissue lacks an antiviral effect.

The reaction to SA shows similarities and differences to the RV response. For the first time in humans, we showed that SA leads to an increase of IL-21, IL-33, IL-22, IFNγ and IFNλ without difference between NP and IT. The deficit in response to SA in terms of reduced IL17 and increased IL-1β release, could be compatible with a more inflammatory reaction in NP than in IT. When nasal mucosa was co-incubated with HRV and SA, the release of IL-6, IFN□, IL-17, and TNF□ was suppressed in both NP and IT. Whereas IL-1β was increased in NP, IFNλ and IL-21 were increased in both NP and IT (Fig. 5).

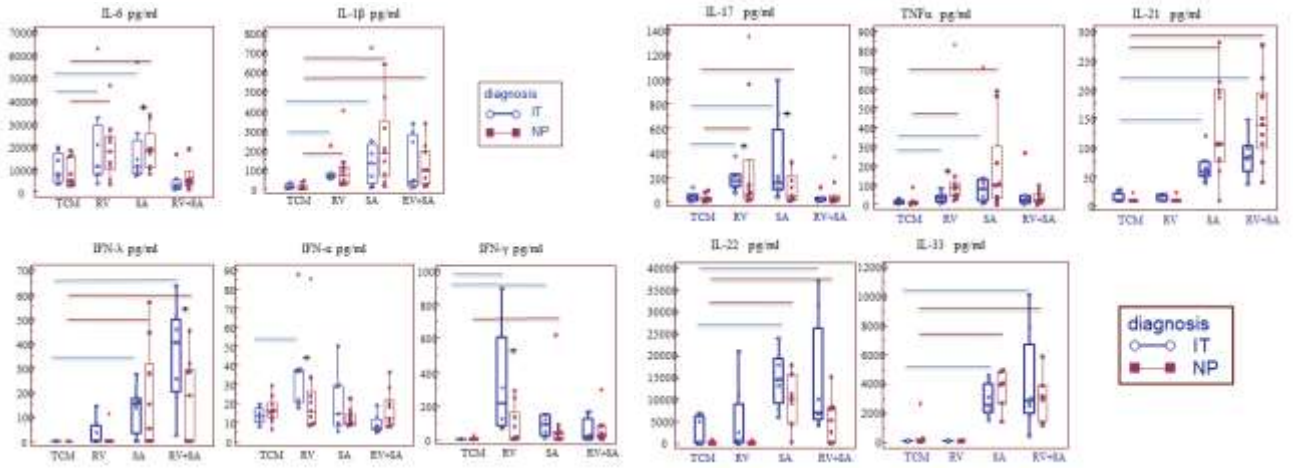

Figure 5 – Cytokine production in Inferior Turbinate (IT) or Nasal Polyp (NP) tissue after 24h exposure to SA, RV or both. Different patterns are observed

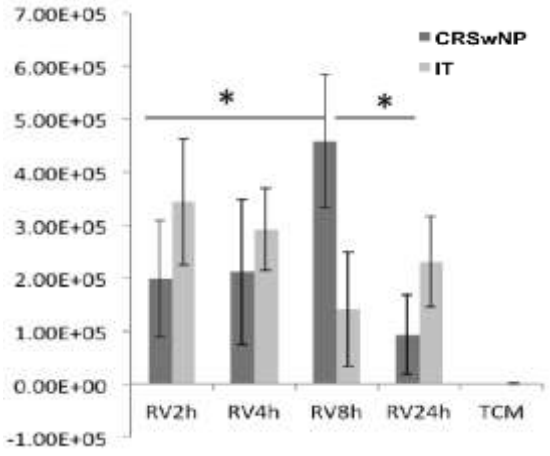

Figure 6 – Replication kinetics of RV is different between polyp and inferior turbinate epithelium

RV can also induce IL-5 in NP but not IT tissue. The replication kinetics of RV was different in IT and NP tissue where its replication peaked at 8h (Fig.6).

SA can also induce the production of IL33 and TSLP from the bronchial epithelial cell line BEAS-2B, as well as in nasal polyp tissue resulting in an augmented Th2 response. This induction can be reduced by targeting toll-like receptor 2. The localization of IL33 and TSLP were also examined in the tissue, as well as the respective receptors. Abundant IL33 was found in the extracellular space between epithelial cells of polyp tissue after SA stimulation. Both IL33 and TSLP receptors were significantly upregulated on CD3+ cells present in the nasal polyp tissue after exposure to SA.

Viral-bacterial interactions were also studied in patient cohorts. Nasopharyngeal samples were obtained from children 3 months to 6 years of age with a clinical presentation of an upper respiratory viral infection and controls and cultured for common bacteria. PCRs were performed for common respiratory viruses.

Children who were symptomatic were significantly more likely to harbor bacteria (86% vs 72%) and more specifically Streptococcus Pneumoniae (37% vs 24%), Moraxella Catarrhalis (43% vs 25%) and multiple bacteria (46% vs 35%) (Fig.7). The highest colonization rates were seen in children with influenza infection. Moraxella colonization was associated with the presence of RV, while Haemophilus with RSV.

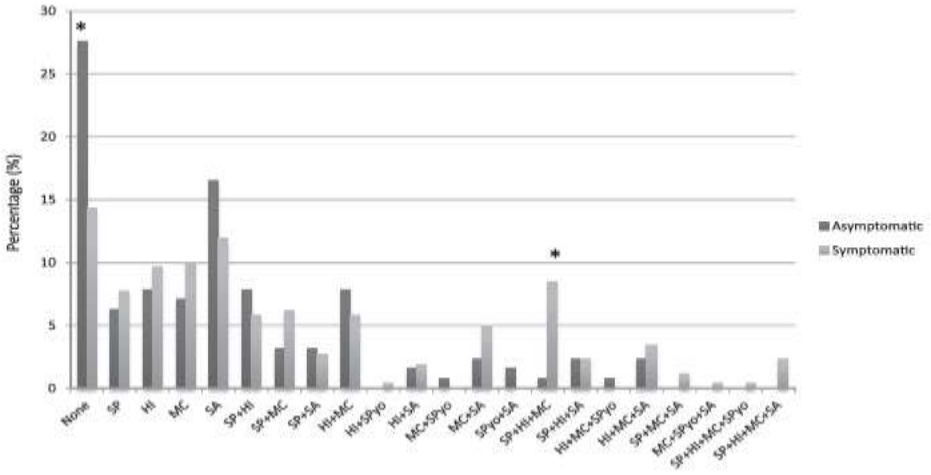

Figure 7 – Contribution of individual bacteria and combinations in the colonization of the upper airways in symptomatic and control children

The respiratory epithelium

The cells that line the airways, the respiratory epithelium, are the first to meet triggers from the outside world and have to respond to them. In fact, it is mostly within the epithelial cells that viruses replicate. Therefore, it appears that the epithelium plays a central role in defending against external factors such as viruses, but also initiates and coordinates the wider response, by sending signals to other elements of the immune system.

The epithelial response to RV has been shown to be deficient in patients with allergic asthma, producing less of the key antiviral molecules: interferons. This problem is not always present and may be related to other factors, such as the severity of asthma or additional triggers.

In PreDicta, we investigated whether the interferon deficiency problem was also present in cells from the nose. The nose epithelium is of course much easier to access and sample, but it also appears to be quite similar in many, but not all, aspects to the lower airway.

We obtained and cultured nasal epithelial cells (NEC) from normal individuals, and people suffering from allergic rhinitis or asthma (allergic or not). We infected these epithelial cell cultures with RV and measured the production of interferons and other molecules, but also how much the virus proliferated and the amount of damage it induced to the cells coming from these different groups of people.

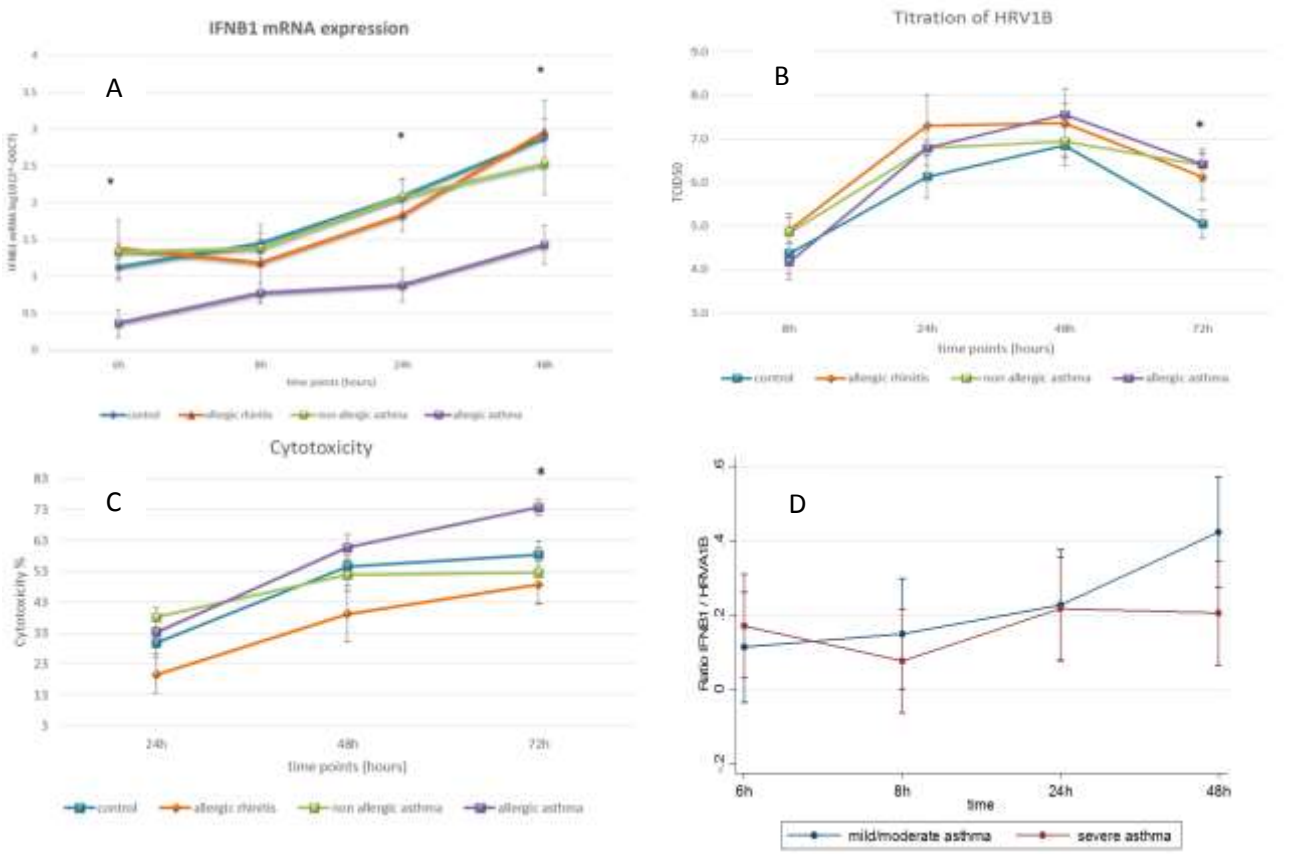

Figure 8

We found that cells from people with asthma without allergy, or allergic rhinitis without asthma had no apparent deficiency in interferon production. However, a clear defect was present in cells from patients with allergic asthma (Figure 8A). These patients had also the highest virus replication and cell damage (Fig 8B, C). The defect was in fact stronger in patients with more severe disease (Fig 8D).

When we directly compared epithelial cells from the nose to cells from the bronchi of the same person, we observed that bronchial cells from allergic asthma were slightly more susceptible than nasal cells, however, the key phenomenon of interferon deficiency was present in both. Therefore, antiviral deficiency in allergic asthma is a wider problem and not only local in the lung. Nasal cells can provide a nice model to study these responses.

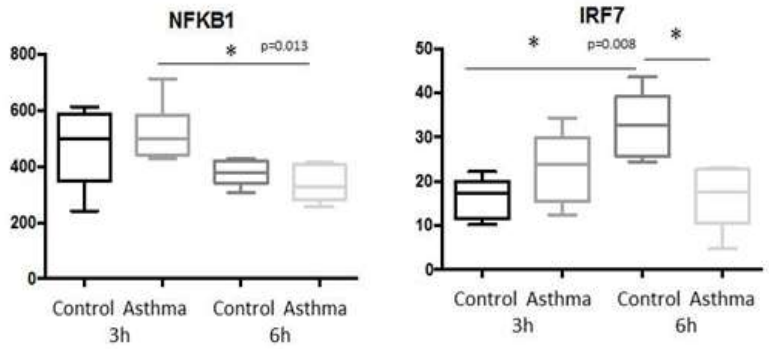

Figure 9 – Levels of expression of the transcription factors NFκB1 (left) and IRF7 (right) in nasal epithelial cells from normals or asthmatics after 3 and 6 hours of exposure or not to RV. The epithelial inflammatory and antiviral response is dampened in allergic asthma

We also took a wider perspective to see the response to the RV infection of all the genes in epithelial cells. We used a method called deep sequencing to measure all the genes that changed during the infection and compared in order to find the ones that are different (differentially expressed genes, DEG) between normal and asthmatic individuals. We decided to explore early points in time to see what happens at the very beginning of the infection. We found that

three hours after the infection starts normal and asthmatic individuals have the same number of genes activated, but asthmatics have many more genes slowing or shut down (downregulated). Because of this early downregulation, at 6 hours, the asthmatic response is very much dampened. Several key genes involved in inflammatory and antiviral responses, such as NFkB and IRF7 are affected (Fig 9).

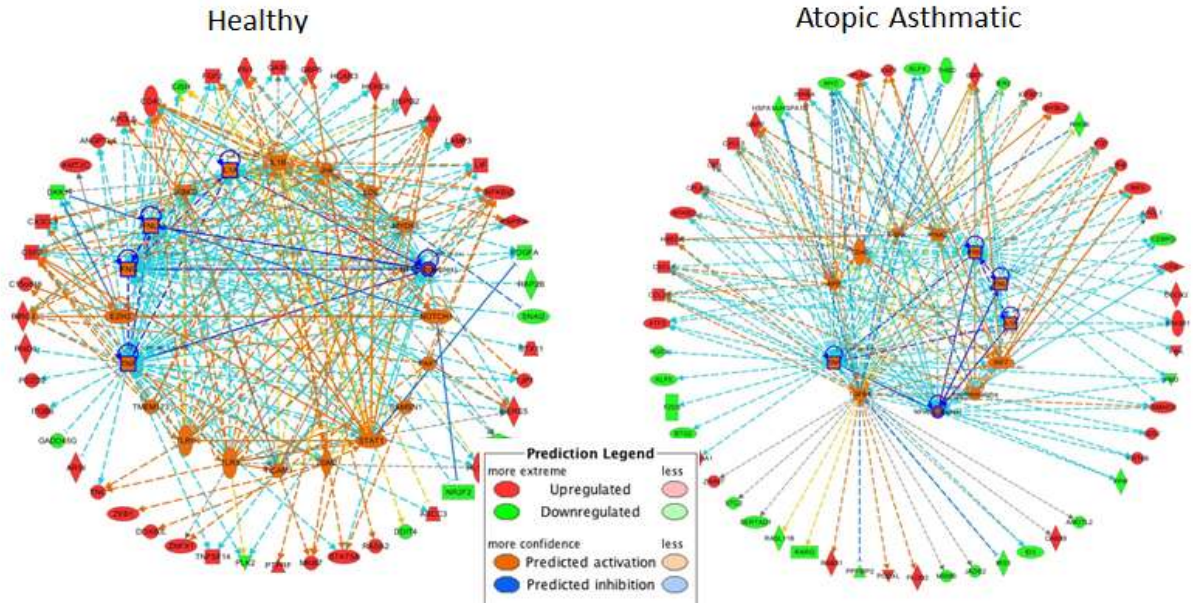

Figure 10 – The inner circle depicts genes that change with RV infection and the outer circle represents the transcription factors that regulate those genes. Over and under regulation are color-coded. An important reduction of the response of the epithelium to RV in asthma can be seen.

The effects of this phenomenon were very obvious when we focused on the factors that regulate the transcription of genes. Transcriptional regulation is much less active in asthma, as shown in Figure10.

So it appears that in contrast to normal epithelial cells, which mount a robust response against the virus, cells from people with allergic asthma are inhibited, therefore giving the opportunity to the virus to replicate more and do more damage.

In parallel to the global overview of the antiviral response, we were also interested in assessing molecules that regulate the production of antiviral interferons, in order to identify possible targets for intervention. Among such molecules, we have found that SOCS-1, a nuclear factor controlling IFN, is overexpressed after infection in cells from asthmatics. SOCS-1 is found in higher levels in bronchial epithelial cells and biopsies from patients with

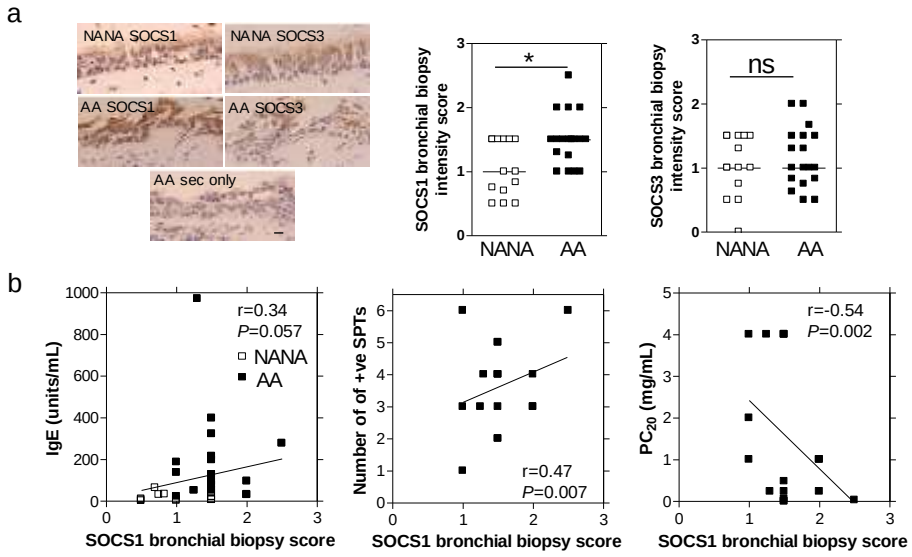

Figure 11: SOCS1 but not SOCS3 protein is increased in bronchial biopsies of atopic asthmatic adult donors and positively correlates with SPT and is related to airway hyper responsiveness. Bronchial biopsies were taken from 18 mild-moderate asthmatic adults (AA) and 12 healthy controls (NANA) and stained for bronchial epithelial SOCS1 and SOCS3 (a). Bronchial epithelial SOCS1 scores were compared with total IgE, number of SPTs and PC20 data (b). Bronchial epithelial SOCS1 scores were associated with atopy and airway hyperresponsiveness

asthma (Figure 11a), while when it is experimentally overexpressed, IFN (beta and lamda) responses are suppressed. The levels of SOCS-1 were also correlated to the number of positive skin prick tests (i.e. level of allegy) and to the level of bronchial hyperresponsiveness (Figure 11b).

As mentioned before, the epithelium is not only central to the antiviral response, but it also regulates and direct the responses that follow, by producing mediators able to shift the type of immune response that follows. We have identified an important role for the epithelial derived pro-Th2 factors IL25 and IL33 in RV-induced asthma: such factors are produced by the respiratory epithelium in response to RV infection and can drive acute exacerbations through, among other, innate lymphoid cell (ILC) production of IL5 and IL13. Their levels correlate to exacerbation severity.

Induced by RV infection and enhanced by allergic (atopic) status, IL-25 binds to the IL-17RB receptor and triggers the activation of cells related to allergic inflammation, such as Th2 and ILC2-like cells. This boosts Th2-mediated pro-inflammatory responses, intensifying disease symptoms and leading to disease exacerbations. At the same time, IL-25 boosts viral load, further contributing to the severity and persistence of exacerbations. Inhibiting IL-25, or its receptor IL-17RB, therefore constitutes a promising therapeutic approach (Fig12).

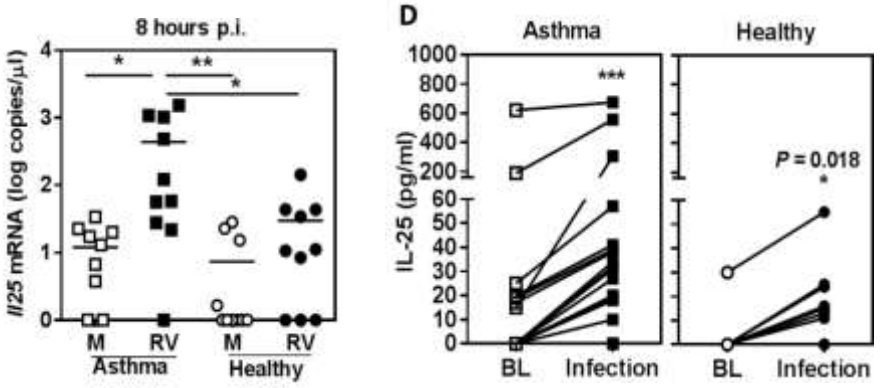

Figure 12: Bronchial epithelial cells from asthmatic patients produce more IL-25 than normal control s (left) Higher levels of IL-25 are also found in the airways of patients after experimental infection with RV (right)

Interleukin-33 (IL-33) is also overproduced by epithelial cells of atopic asthmatics. When immature (Th0) T-helper cells and innate lymphoid cells (ILC) were incubated with supernatants of RV-infected epithelial cells, these cells developed a more type 2 (allergy-related) phenotype, producing high levels of IL-4, IL-5 and IL-13. This reaction almost completely stopped when the receptor for IL-33 was blocked, suggesting that IL-33 is required to drive the allergic inflammation after RV infection (Fig 13).

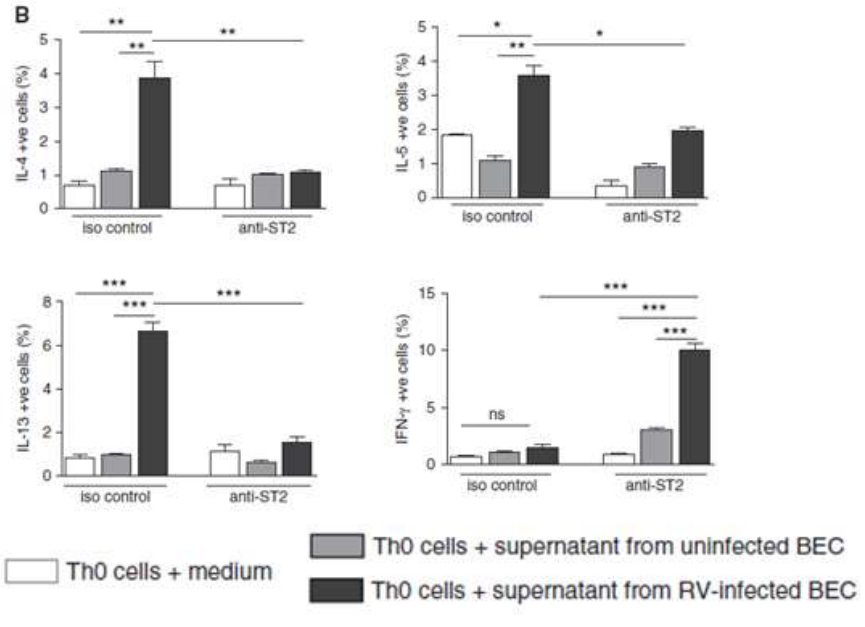

Figure 13 –When naïve Th0 cells are exposed to supernatants of epithelial cells infected or nor with RV, there is overproduction of Th2 cytokines (IL-4, IL-5, IL-1), but not IFN-γ. Blocking the IL-33 receptor ST2, reverses the cytokine pattern

In other experiments, we have measured the levels of important mediators produced by the epithelium after RV infection in cells from different subject groups. In addition to IFN-β, also IP10, RANTES and TNFα, came up as important mediators that are differentially regulated between the asthma and normal state.

Immune regulation

The generation and maintenance of allergen-specific T-cell tolerance is a key step in healthy immune responses. Breaking of peripheral T-cell tolerance to allergens can lead to the development of allergies. We have shown that triggering of Toll-like receptor (TLR)4 or TLR8 and the proinflammatory cytokines IL-1β or IL-6 break allergen-specific T-cell tolerance in human tonsils and peripheral blood through a mechanism dependent on the adaptor molecule MyD88. Myeloid DCs and stimulations that activate them such as TLR4 and TLR8 broke the tolerance of allergen-specific CD4+ T cells, whereas plasmacytoid DCs and stimulations that activate them, such as TLR7 and TLR9, did not have any effect (Fig14). Tolerance breaking conditions induced by different molecular mechanisms were associated with a mixed cytokine profile with a tendency towards increased levels of IL-13 and IL-17. This breaking of tolerance after exposure to signals that can be associated to microbial stimuli is an important step in understanding how infection may induce allergy.

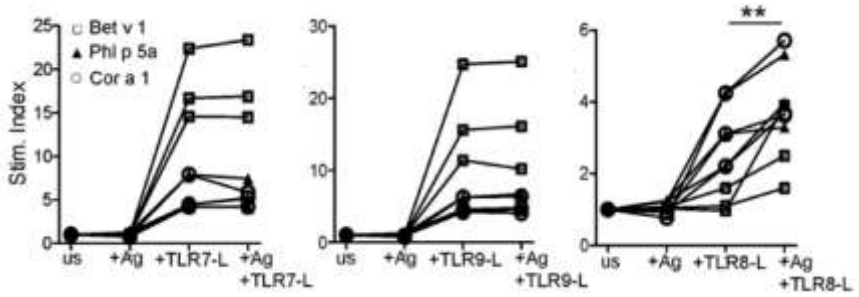

Figure 14 – Tonsil cells from allergic individuals were cultured with different allergens and with or without the addition of a TLR stimulus. When TLR8 is added to the allergen there is higher proliferation, showing that tolerance is broken

We have studied human tonsils as an important immune organ. Regulatory (FOXP3+) T-cells and plasmacytoid dendritic cells (pDCs) were identified in the T-cell areas of the tonsils where they co-localised in the proximity of crypt epithelial cells (Fig. 15)

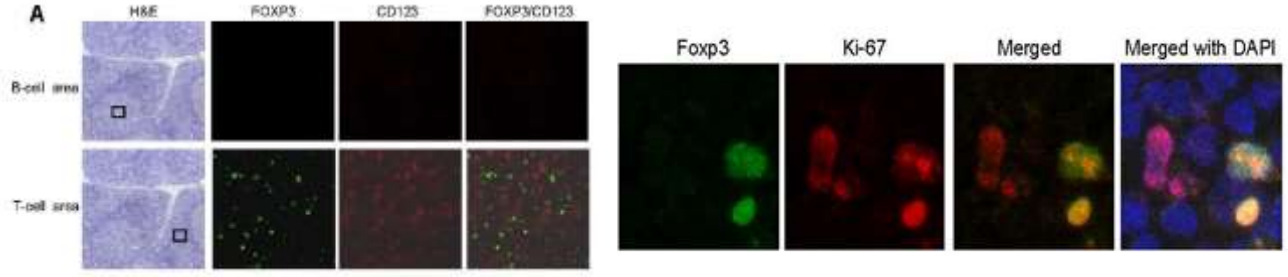

Figure 15 – A: The black squares indicate the B and T cells area of the tonsil. Sections were stained for FOXP3 (Treg cells) and CD123 (pDCs). Furthermore approximately 40% of the T-reg cells in the tonsils were proliferating (Ki-67), showing that tonsils are a suitable niche for the in-vivo expansion of regulatory cells

Asthma exacerbations are most often associated with viral infections, however, allergen exposure can also be a factor, either alone or in synergy with the viral trigger. Whether the immune responses leading to each of these conditions is similar or distinct has not been known. We used multicolor flow cytometry to

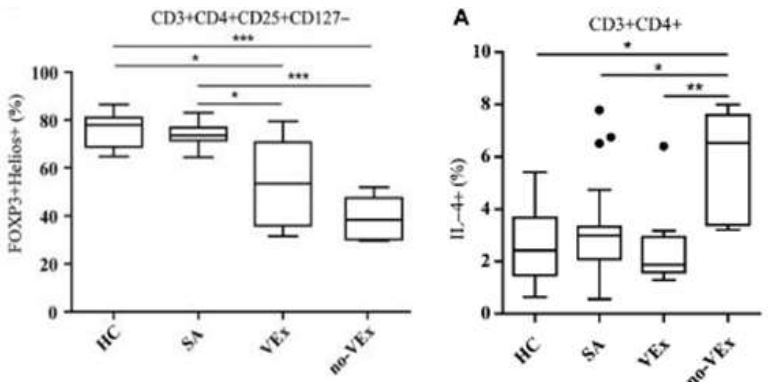

Figure 16

evaluate phenotypical changes in CD4+ T cells in subjects during an exacerbation and at the steady state. Viruses were also detected at the time of the exacerbation. It was apparent that Treg cells change significantly during exacerbations with their number reduced significantly in peripheral blood. When comparing exacerbations associated with virus infection and others associated with other stimuli, two distinct patterns in relation to T-regulatory cells were revealed. A depression of T-regs was observed in patients with exacerbation, which however was significantly stronger in those with exacerbations not associated with viruses. The cytokine profile of exacerbations changed from stable asthma but also with different apparent trigger: virus-induced exacerbations had significantly lower intracytoplasmic IL-4 in their CD3+CD4+ cells. However, the expression of IL-17 in CD3+CD4+ cells was upregulated in all exacerbations in comparison to baseline (Fig 16).

Next we studied the RV ability to attach and enter to the lymphocytes as the first events needed for the infection, by labeling RV with fluorescent dye and performing flow cytometry. RV attached and probably also entered into the monocytes already 30 minutes and to CD4+ T and B lymphocytes 8 hours after the addition of the virus. Monocytes were almost 100% positive for both UV-treated and untreated RV after 24h. This indicates that in monocytes the virus was taken up via passive internalization. Remarkably, a small proportion (3%-15%) of T cells and B cells were also detected to be positive when the higher virus concentration was used. This was confirmed with imaging flow cytometry (Fig. 17) CD8 cytotoxic T-cells did

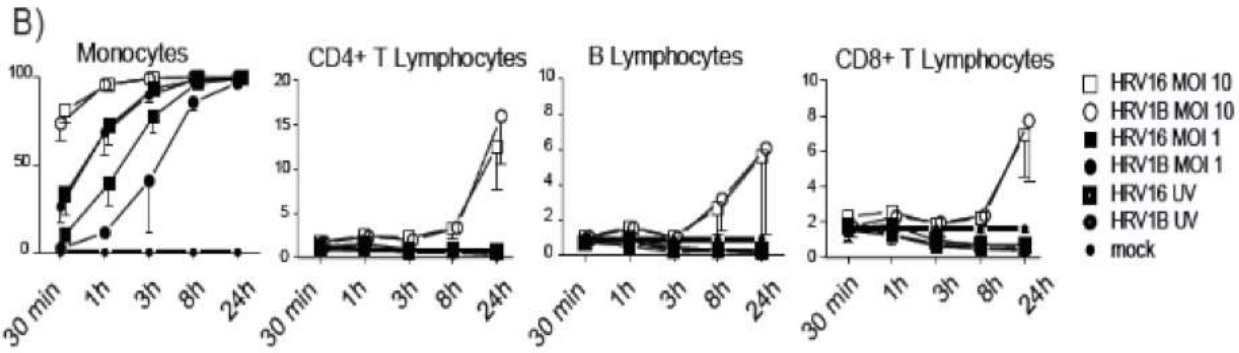

Figure 17 – Different fluorescent-labeled RVs were incubated with lymphocyte subsets and the association of virus particles with cells was evaluated. Monocytes uptake ether live or inactivated virus from very early time point. Live virus particles appear in association with T and B cells after 8 hours

not give a positive signal, while in CD4+ T cells RV was seen on cell surface where it co-localized with anti-CD4. In monocytes and CD19+ B cells, however, the staining pattern indicates that the virus was located inside the cells. Monocytes internalize both live and UV-inactivated viruses, suggesting a passive event. In contrast, in B-cell the virus appears after 8 hours after, while UV-inactivated virus cannot enter.

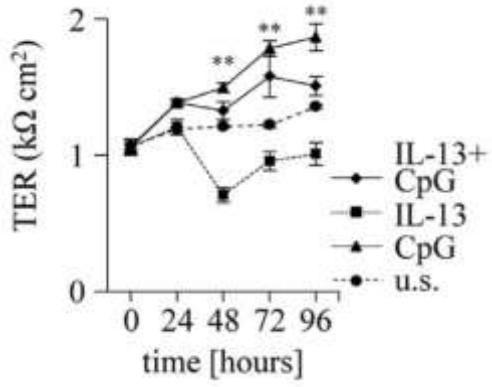

Figure 18 – Transepithelial resistance is compromised by IL-13, but improved by CpG. Moreover, CpG may counteract the IL13 effect

Investigating the effects of immune responses on the epithelial barrier, we have found that CpG-DNA, which is recognized by Toll-like receptor (TLR) 9, enhances barrier function of bronchial epithelial cells by increasing tight junction (TJ) molecule expression. When we stimulated air-liquid interphase (ALI) cultures of primary human bronchial epithelial cells from non-asthmatic subjects with CpG-2006, transepithelial electric resistance (TER) was increased and paracellular permeability, measured as diffusion of FITC–conjugated dextran, was decreased in a dose-dependent manner. It was demonstrated previously that the Th2 response, particularly when mediated by IL-4 and IL-13, could compromise the epithelial barrier. IL-13 decreased while CpG-2006 increased barrier integrity in ALI cultures. Of interest, CpG-2006 treatment rescued the decrease in IL-13–stimulated ALI-cultured bronchial epithelial cells (Fig18). However, when bronchial epithelial cells were pretreated with IL-13, CpG-2006 could not overcome the barrier impairment suggesting that the chronic

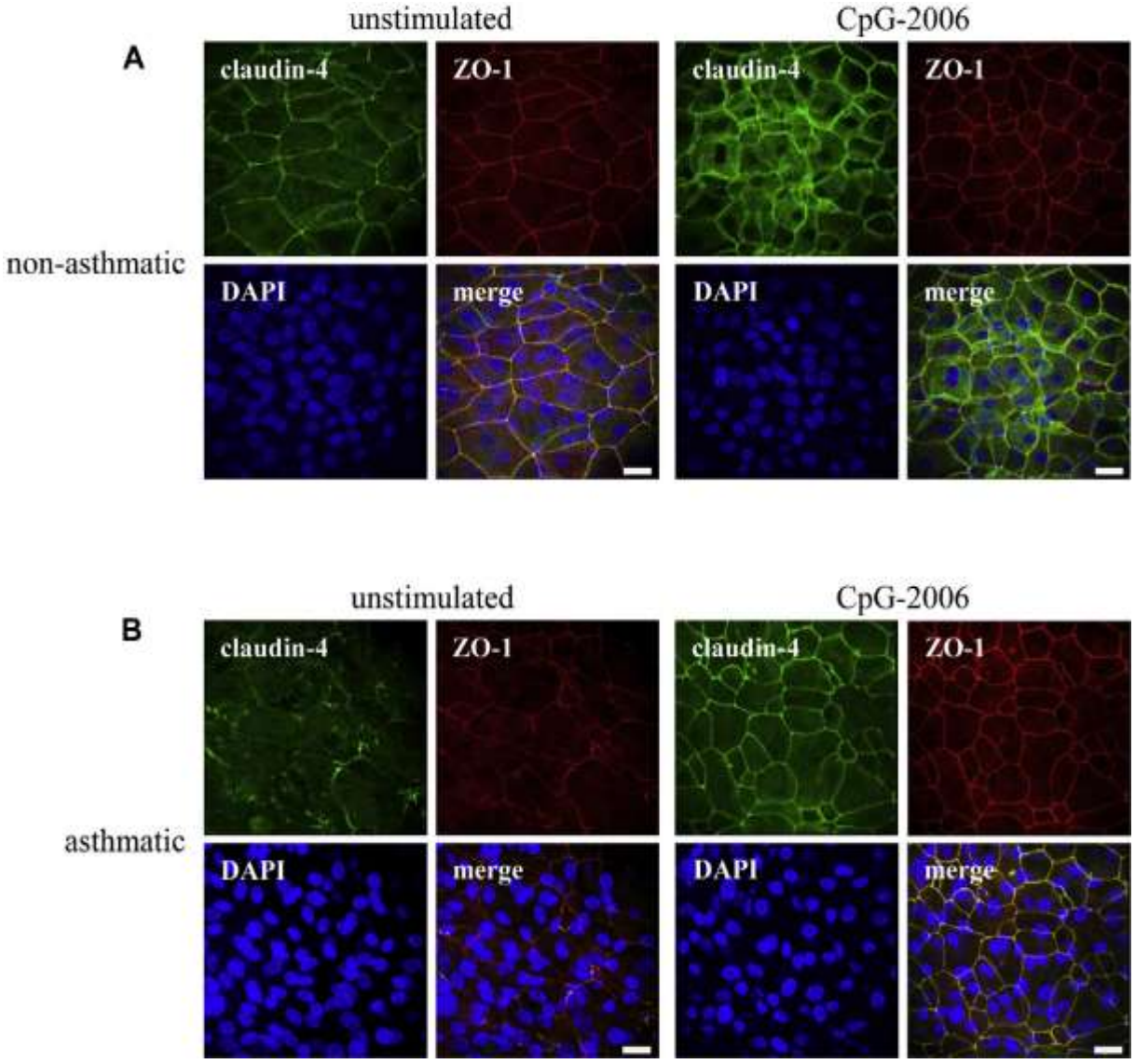

Figure 19 - Immunofluorescence detection of claudin-4 (green) and ZO-1 (red) in ALI-cultured bronchial epithelial cells from nonasthmatic (A) and asthmatic (B) subjects

status or severity of the type-2 immune response during inflammation can influence the efficacy of CpG. Because TJ proteins must be correctly assembled into TJ structures to efficiently contribute to the barrier function, we investigated the distribution of the TJ-associated molecule ZO-1 and claudin-4 protein, which is known as a sealing claudin. CpG-2006–stimulated bronchial epithelial cells from non-asthmatic donors had higher ZO-1 and claudin-4 immunofluorescence at the cell boundary compared with unstimulated control cells (Fig 19, A). Cells from asthmatic patients had decreased claudin-4 and ZO-1. CpG-2006 stimulation remarkably restored this impaired (Fig 19, B). Therefore improvement of the barrier in bronchial epithelial cells by CpG-2006 seems to be mediated by enhanced expression of TJ-related molecules and their appropriate distribution on cell-cell borders. In conclusion, these data suggest that administration of CpG-DNA could be a useful intervention and demonstrate an additional explanation for the hygiene hypothesis in both the prevention and treatment of asthma by restoring impaired epithelial barrier.

Antibodies against Human Rhinovirus

One of the key objectives of PreDicta was to design a diagnostic chip for monitoring the antibody responses to different RV groups. Little is known about these responses: in general terms antibody responses to RV develop after several weeks, so they don't influence a current infection, but they protect from a subsequent one with the same strain. Understanding the antibody responses is a prerequisite for the design and development of effective vaccines. Furthermore, antibody monitoring may give us information about previous exposures and reactions to RV, as well as 'signatures' from different RV strain groups that may be associated with future outcomes.

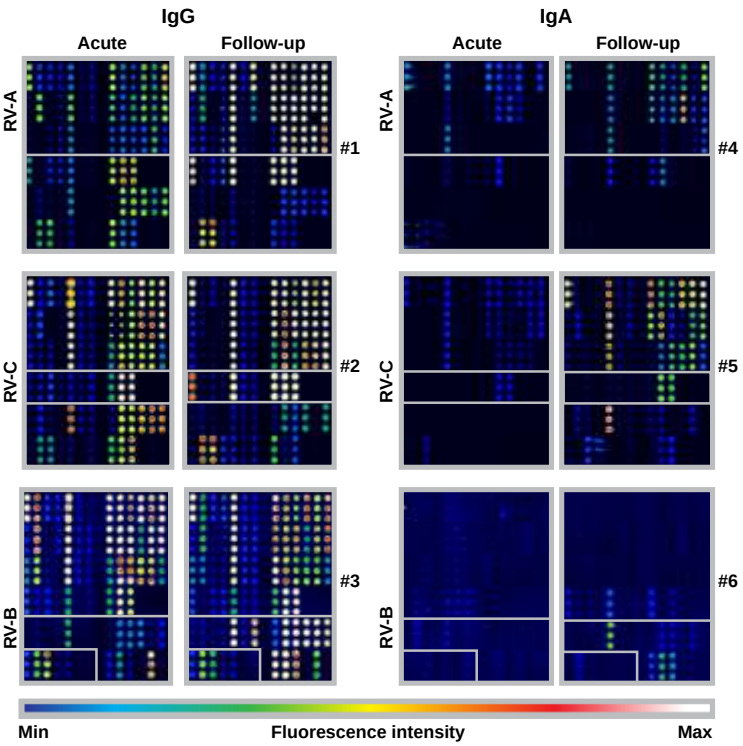

Figure 20. Antibody reactivity profiles to micro-arrayed RV antigens. Images obtained by testing serum samples from 6 representative patients (#1-6) who attended the hospital due to an acute episode of airway obstruction. Measured were IgG (*left panel*) and IgA (*right panel*) reactivities. Increasing fluorescence intensities from blue to red/white correspond to the amount of bound antibodies. RV proteins and peptides of the culprit RV group are boxed.

After isolating and purifying different RV proteins and peptides and testing their ability to bind with antibodies, we have spotted 130 components of RV on a glass chip and optimised its use. The resulting chip is an important new tool that can be used in many different ways and settings and brings RV antibody research to a new era (Fig.20). Already several interesting finding are reported below.

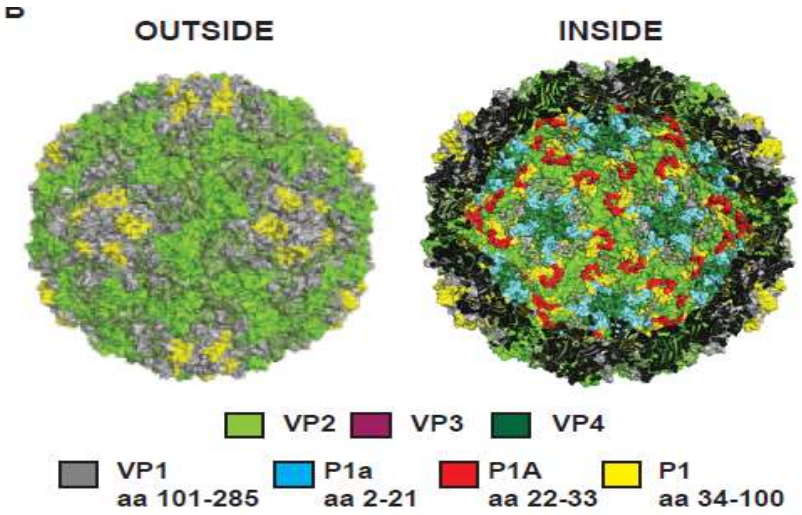

Figure 21 – We have identified that the part of the virus where most of the antibodies bind is P1A, part of the VP1 protein (shown in red). Interestingly, P1A appears to be expressed only in the inside part of the virus capsid, suggesting that this is a mechanism by which the virus evades immune surveillance

In parallel to the development of the chip, we explored different aspects of the antibody responses to RV. In order to map the sites where RV antibodies target, we have synthesized proteins from different parts of the virus. VP1, which is also the site of receptor binding, was the main target for the memory immune response with IgG1 and IgA antibodies. Sera from children with a recent RV infection were used. Interestingly, this response was mainly against one part of the VP1, close to its N-terminal (P1A), that becomes exposed only when the virus binds to its ICAM-1 receptor. Molecular modeling using the 3-dimensional RV capsid structures revealed that P1A was localized inside the capsid and outside the areas involved in receptor binding or RV neutralization. Our results suggest that the virus misdirects the immune system to produce an antibody against an epitope that is not protective, as a mechanism to escape immunity and cause recurrent infections (Fig.21).

Subsequently, we evaluated the antibody responses in human adults following an experimental infection with RV16 as well as in a mouse model.

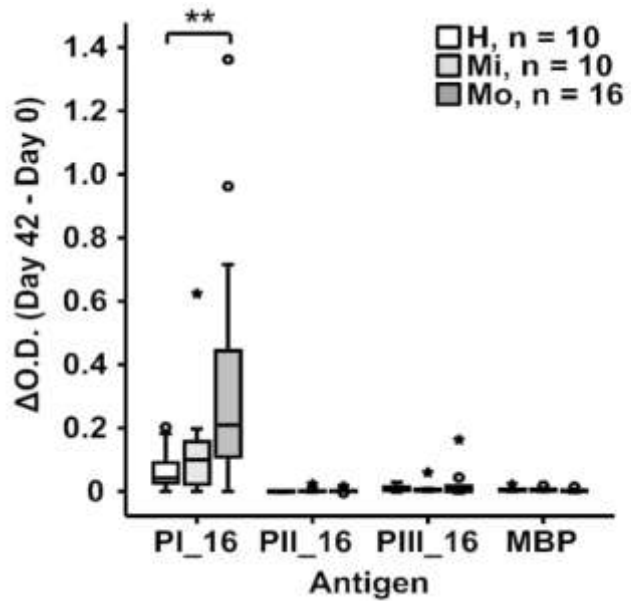

Figure 22 - Shown are IgG1 increases between days 0 and 42 for different virus proteins in the three groups (healthy, mild and moderate asthma).

In the experimental infection setting, volunteers either healthy or with a diagnosis of asthma, were exposed to RV16, in the context of a previously reported study. Serum was available both before the experimental infection and six weeks later during convalescence. We measured antibody responses of different subclasses to different RV antigens. RV antibody levels were higher in asthma than in controls. Six weeks after infection, IgG1 antibodies showed a group-specific increase towards the N-terminal VP1 fragment, but not towards other capsid and non-structural proteins. Patients with severe respiratory symptoms show higher increases of PI_16-specific antibody levels than patients with fewer symptoms (Fig.22). These results demonstrate that increases of antibodies towards the VP1 N-terminus are group-specific and associated with severity of respiratory symptoms.

Using sera from children with an acute wheezing attack, we have analysed the patterns of antibody responses against different virus subgroups. We first prepared a phylogenetic clustering of the different peptides used in the chip. These peptide homology groups, represented to a large extent the RV subgroups

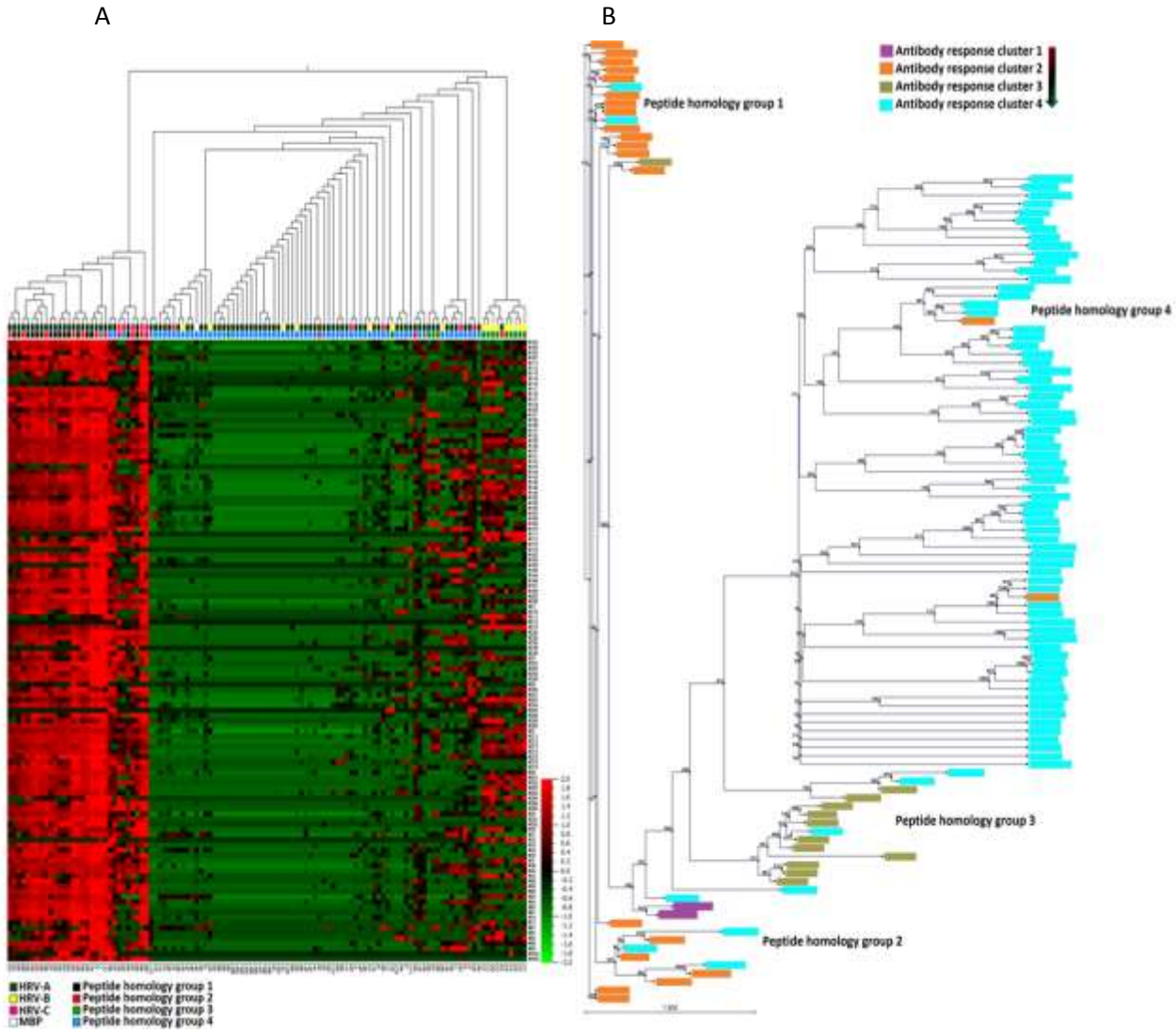

Figure 23. Heatmap representation of RV-specific antibody responses measured by RV chip. A) Shown are IgG levels to 128 RV micro-arrayed components measured in serum samples from 120 children with an acute wheezing attack. Red represents high and green low values. Rows are peptide-specific antibody responses for each subject and columns are antigen-specific antibody responses throughout all subjects. The color-coded annotations on the top side of the heatmap represent pre-determined groups of peptides that refer either to the RV group (A, B, C) or the peptide homology group as determined in B. B) The peptides' amino acid sequences were aligned and based on the alignment, a homology distance cladogram was built. The peptides were then color-coded based on the antibody response cluster that they belong to, with purple representing the cluster with the highest mean measurement and light-blue the cluster with the lowest mean measurement.

A, B and C. Then we used an unsupervised computer algorithm to cluster the patterns of antibody responses. Finally we superimposed the results of these analyses. As it is shown in Figure 23B, there is a very strong correlation between the two groupings, showing that antibody response patterns reflect very closely the peptide structures. We are now using the results from the chip to compare the antibody response in normal versus asthmatic children in the pediatric cohort of PreDicta, as well as changes of antibody responses over time in the same children.

In the mouse model, we studied the induction, magnitude and specificity of antibody responses. It is important to know whether infection with one RV strain may result to some protection towards other

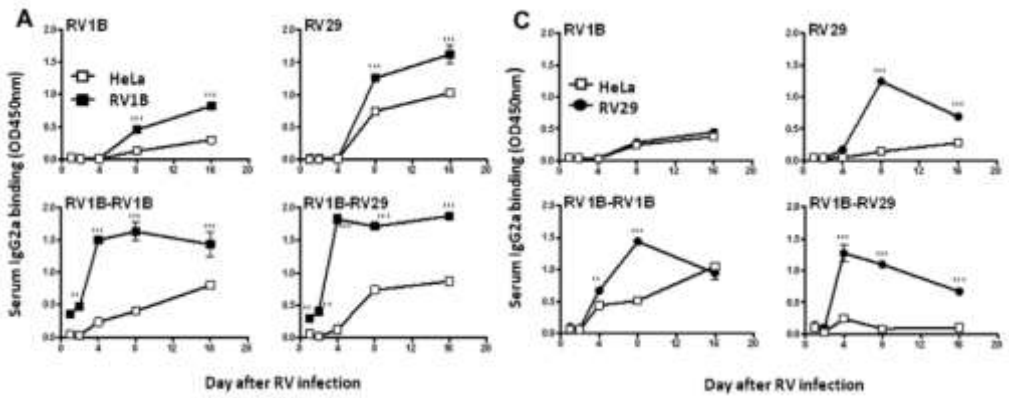

Figure 24 – Mice were infected with RV1b or control (HeLa) and reinfected with either RV1b or RV29 after a month. Antibody (IgG2a) responses against purified RV1b (A) or RV29 (C) are shown

strains as well, for the development of effective, cross-reactive vaccines. In mice, strong cross-serotype RV-specific IgG responses in serum and bronchoalveolar lavage were induced towards the RV capsid protein VP1. IgA responses were weaker, requiring two infections to generate detectable RV-specific binding. Similarly two or more RV infections were necessary to induce neutralising antibodies. Immunisation strategies boosted homotypic as well as inducing cross-serotype neutralising IgG responses. Therefore, the possibility of generating an antibody response against more than one serotype is realistic, but requires strong stimulation: this may be overcome with the use of adjuvants.

Resolution of inflammation

Recent findings indicate that the resolution of inflammation does not occur spontaneously or passively, but it is most probably an active process, mediated at least in part through lipid molecules, which are part of the arachidonic acid pathway. In order to study the active mechanisms of inflammation resolution, we have developed and optimized a new methodology based on liquid chromatography/mass spectrometry, capable of measuring accurately, with sensitivity and in parallel, several lipid mediators which have been associated with inflammation and its resolution (Fig.25). These include Lipoxin A4 (LXA4), Resolvin D1 (RvD1), Resolvin E1 (RvE1), Protectin D1 (PD1) and its stereoisomer 10S,17S-DiHDoHE (PDX), Prostaglandin D2 (PGD2), Leukotriene B4 (LTB4) and Eoxin C4 (EXC4). The methodology was validated using human and mouse sera, mouse lung tissue and culture media, thus enabling fast and accurate determination of lipid mediators in these matrices.

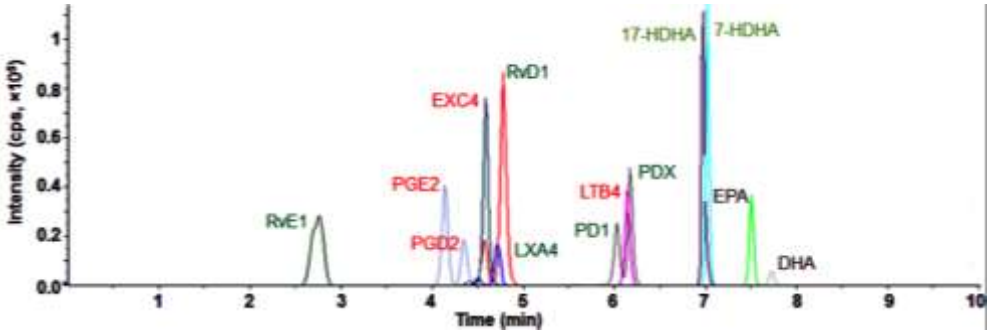

Figure 25 - Advanced LC-MS/MS methodology. Lipid mediators (LM) of different families and functions have been incorporated in the above method.
Colour Code : red = pro-inflammatory LM, dark green = pro-resolving LM, light green = mono-hydroxy intermediates of the DHA metabolism, brown = ω3 polyunsaturated fatty acids

We then used the developed methodology to study the kinetics of inflammation resolution in mice sensitized and exposed to ovalbumin (OVA) allergen. The HDA/17-HDA/PD1-PDX axis was selectively activated when mice were challenged with allergen. Increasing levels of these bioactive mediators correlated with the resolution of inflammation in the model, which starts by day 17 (Fig.26).

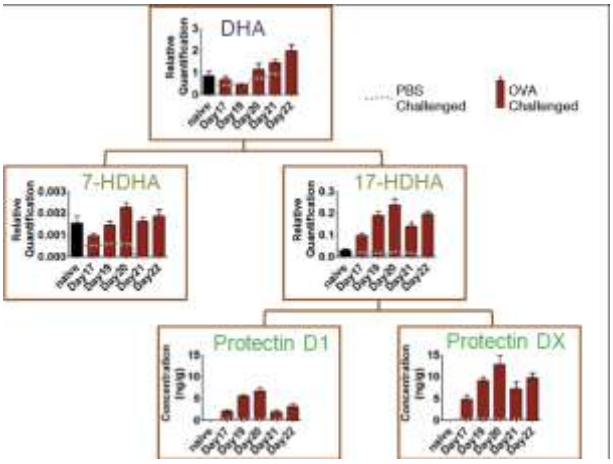

Figure 26

We then assessed the functionality of some of these molecules. Protectin D1 (PD1) and Resolvin D1 (RvD1) were administered to mice in the context of an allergic airway inflammation protocol. Both PD1 and RvD1 were able to reduce cellularity, in particular eosinophils, and reduce the levels of th-2 cytokines. These findings are quite promising about the potential of pre-resolution molecules as therapeutic agents.

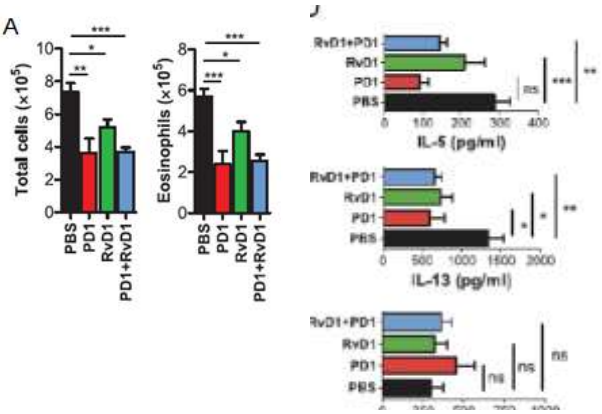

Figure 27

In the mouse model, we went ahead and showed that the anti-inflammatory effects of dexamethasone, a representative corticosteroid, were partly mediated by an early induction wave of the DHA-17-HDHA-PD1/PDX pro-resolving pathway. This induction was evident as soon as 6 hours post dexamethasone treatment, which provided an early priming of the resolution machinery thus enabling faster and more efficient clearance of inflammatory cell infiltrates in the airways and tissue (Figure 28).

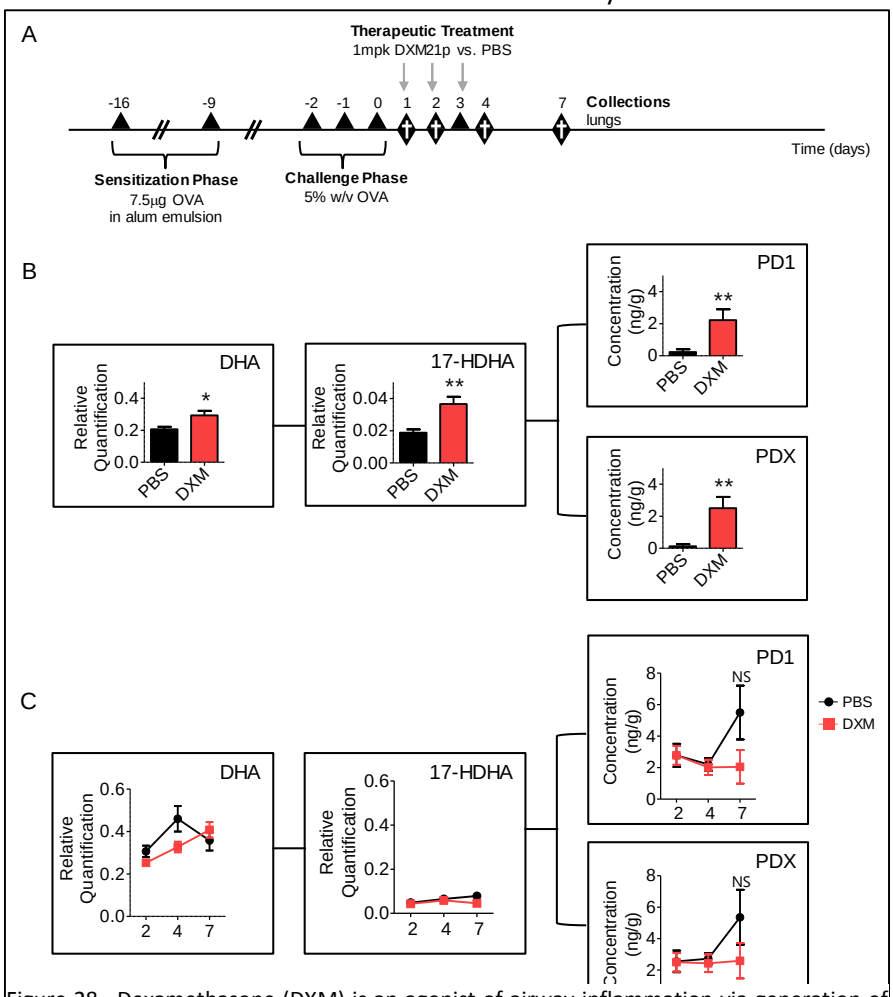

Figure 28 –Dexamethasone (DXM) is an agonist of airway inflammation via generation of protectins. (A) Schematic of the protocol followed for the induction of allergic airway inflammation and DXM treatments. On day1 6 hrs post-dosing levels of pro-resolving D-series protectins were found significantly increased (B) due to increased mobilization of the DHA pathway. (C) LM levels were normalized in the following time-points.

We also measured lipid mediator level in sera from preschool children either normal or suffering from asthma, at baseline, during and exacerbation, at convalescence and at follow-up timepoints. Higher levels of LM were found in the asthmatic children in comparison to normal controls at baseline (Fig 29 left panel).

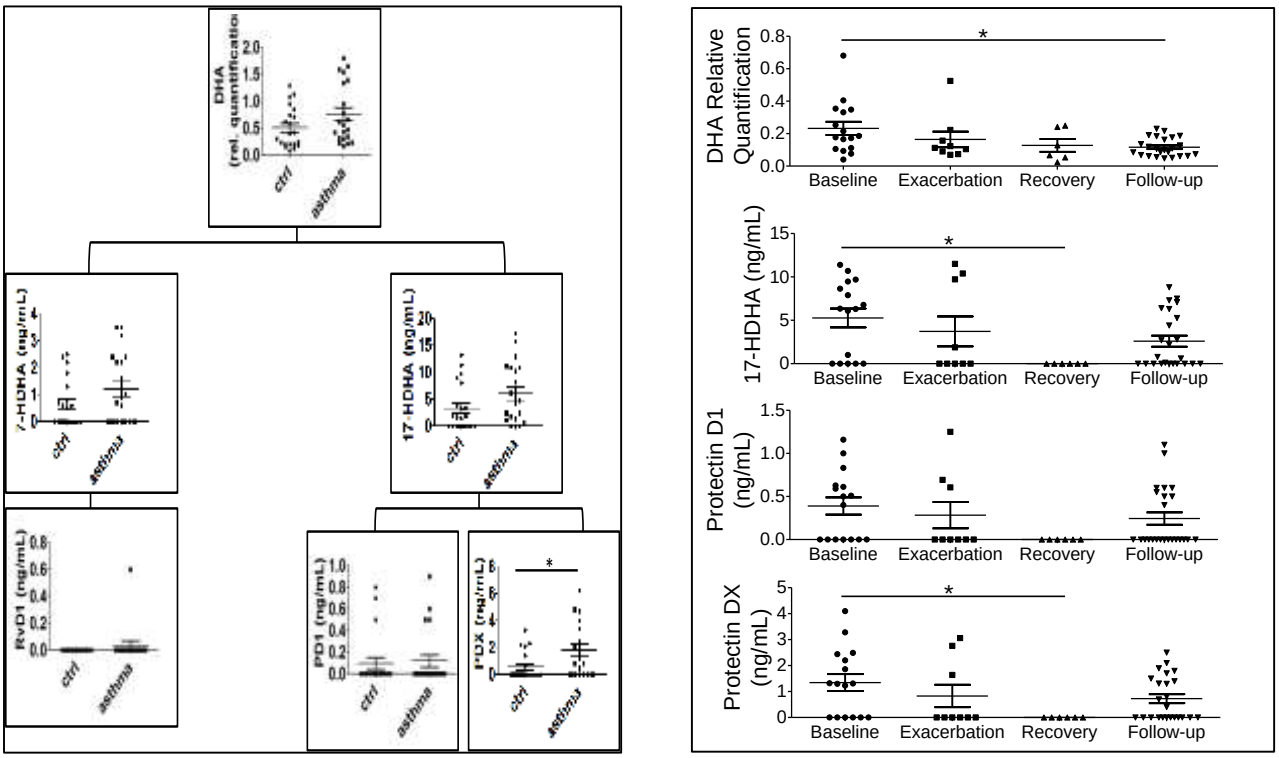

Figure 29

Interestingly, a consistent patent in which LM levels were found reduced in serum during exacerbations and were completely consumed 4-6 weeks later at recovery, was observed. Levels, returned to baseline levels in the follow-up visits (Fig. 29 right panel).

The above observations suggest that there is active generation and consumption of pro-resolvin molecules during acute inflammatory events in the tissues, with delayed kinetics, requiring several weeks to reach a balanced state.

This idea is also compatible with findings in human nasal polyps, in which high levels of LXA4 were associated with disease severity, while the kinetics was reversed in serum (Fig. 30).

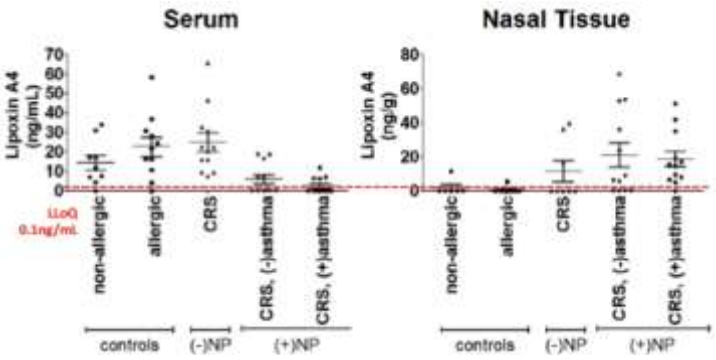

Figure 30

An intervention strategy using antisense technology

One of the major targets of PreDicta was to develop novel therapeutic agents against RV. We have chosen antisense technologies and among them our priority was to develop DNAzymes, a new class of antisense molecules, which consists of DNA molecules that have the capacity of cleaving RNA in specifically targeted

sequences. Very recently, a DNAzyme against the transcription factor GATA-3 was shown to be safe and effective against asthma in humans.

In order to be a good candidate for a therapeutic agent against RV, a DNAzyme should be able to cleave most if not all rhinovirus sequences, with high efficacy. After several attempts and exploring hundreds of different possibilities, we were able to identify a number of DNAzymes. However, these were either very efficient in cleaving, but narrow in the number of RV serotypes they could cleave, or wide in their scope, but not potent enough (Fig 31). Therefore, in order to overcome this problem, we have systematically explored different modifications in the DNAzyme molecules, by expanding or condensing their sequence (Fig 32). After several such experiments, we have managed to identify a number of DNAzymes that fulfill both criteria of efficacy and wide coverage of serotypes.

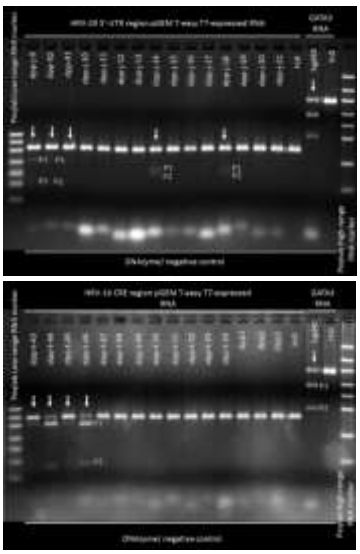

Figure 31 – Examples of DNAzymes with wide coverage but weak cleavage (upper panel), or strong cleavage, but narrow coverage (lower panel)

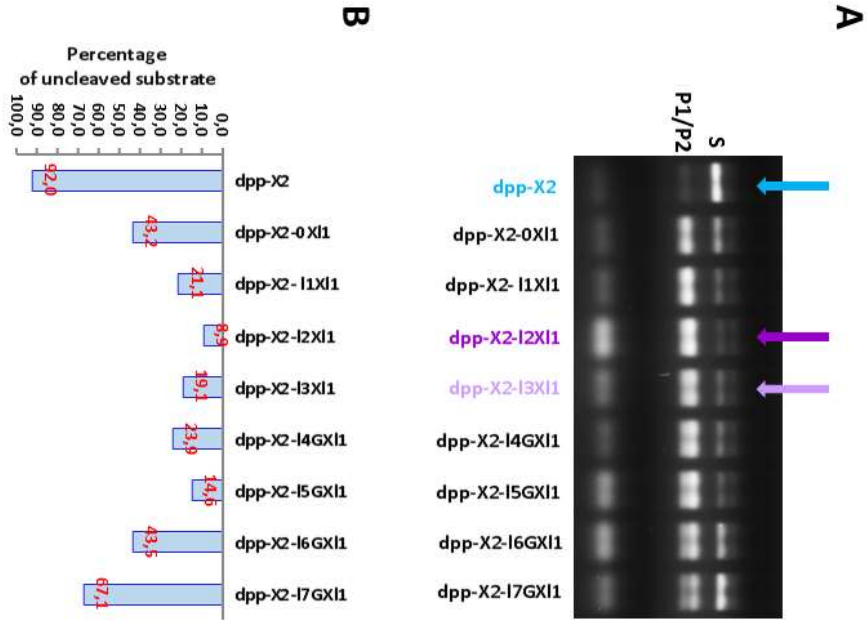

Figure 32- Expanded testing of anti-RV binding arm's length variants exemplified by one of the experiments using dpp-X2 and RV-1b partial RNA. ***Panel A*** shows the raw electrophoretic picture, the densitometric analysis results of which are given in ***Panel B***. The original DNAzyme dpp-X2 is written in blue, while its first length variant we found to show cleavage improvement is written in light violet. The best-cleaving DNAzyme is written in dark violet.

The efficacy of these DNAzymes was then evaluated in-vitro using respiratory epithelial cells infected with RV. A consistent reduction of about 30% was observed in initial experiments (Fig.33). Moreover, we have examined whether these DNAzymes have toxic or off-site effects in cellular models and concluded that there were not. This is a very positive outcome. While several more steps need to be taken before DNAzymes move to a clinical trial, these new molecules hold promise in becoming a new tool against RV and its consequences, including asthma exacerbations.

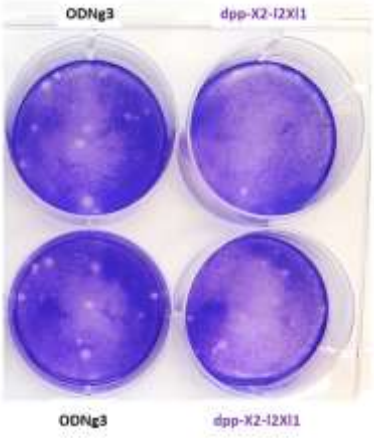

Figure 33 – A plaque assay evaluating the efficacy of DNAzymes against RV in-vitro. Left: control DNAzyme, Right: aRV DNAzyme. A reduction in the number of virus plaques (spots) can be observerd

Although DNAzymes do not require carriers for delivery, for siRNA oligonucleotides this is a prerequisite. We have therefore developed several novel liposomal carriers and optimized them for size, stability, and in-vitro knock-down efficacy in cell culture and in vivo in the lung.

For selected liposomal carriers identified as most effective in cell culture, the process of 'large scale production' was further optimized. The lead formulations were subsequently tested *in vivo* with interesting results. Depending on the formulation used, siRNA could be targeted to the bronchial epithelium or achieve a broader distribution by also transfecting lung macrophages and DCs. These novel siRNA carrier systems are safe, non-immunogenic and effective in mediating target knock-down (Figure 34C), thus offering unprecedented opportunities for performing target validation studies in the lung and developing novel antiviral therapeutics.

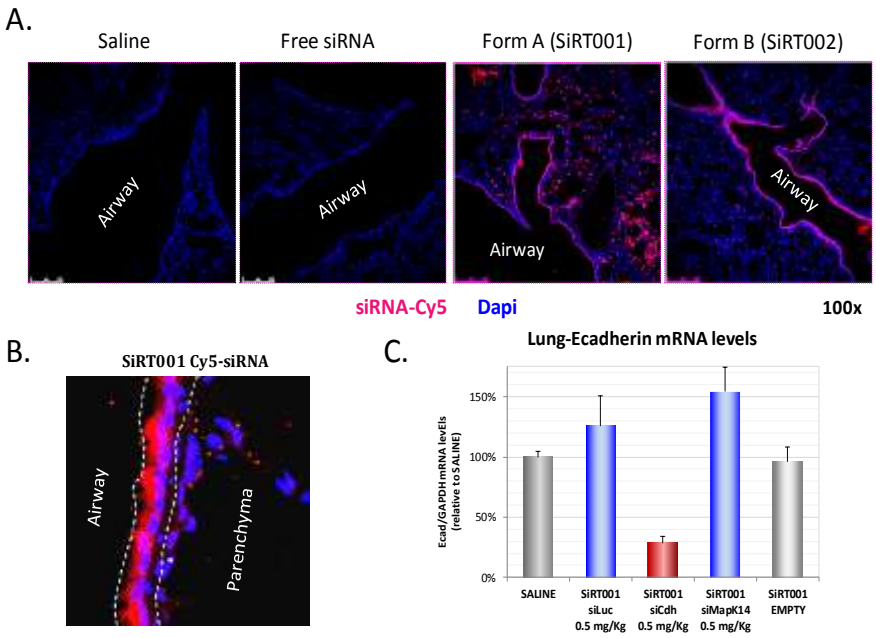

Figure 34 - Novel liposomal carriers were developed that enable the effective and targeted delivery of siRNA molecules to the lung epithelium of mice. (A-B) Biodistribution of labeled siRNA unassisted (free) or encapsulated in formulation A (SiRT001) or formulation B (SiRT002). (C) Target knockdown achieved by SiRT001- formulated siRNA against E-cadherin.

Exploring the mechanisms of RV infection-induced inflammation in-vivo

Our initial intention was to explore the role of multiple RV infections in mouse models. We showed that RV infection induces cross-serotype reactive IgG in serum and secondary infection with either the same or an alternative serotype caused a more rapid and greater magnitude of RV-specific IgG; multiple infections also facilitated IgA and neutralizing antibody responses that were not seen with a single infection. However, it was found that a previous infection, despite inducing robust antibody responses, did not provide protection against re-infection with the same serotype, whereby there was no difference in lung virus RNA levels following secondary vs primary challenge. However, this is not the case in humans, where high levels of antibodies are associated with protection from re-infection with the same strain. In addition we observed an acute Th2 type response upon reinfection, which, we have eventually attributed to the albumin present in the virus preparation. It was shown that airway eosinophilia depended in addition to RV replication also to high levels of contaminating BSA. We concluded that the inflammatory pattern does not represent the human condition and decided to focus on exacerbation models of single virus infection together with allergen.

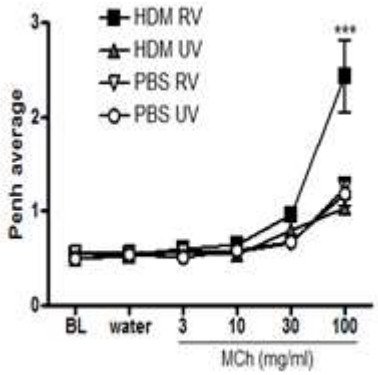

Figure 35 - **AHR in the HDM rhinovirus induced asthma exacerbation model.**
Mice were sensitized i.t. with HDM extract or PBS control and challenged i.n. with HDM and either RV1B or UV-RV1B. 24hrs after infection AHR was measured with methacholine challenge.

An exacerbation model employing exposures to house dust mite (HDM) and subsequently rhinovirus infection demonstrated that increased airway hyperresponsiveness develops after the combination of exposures, rather than each factor alone (Fig. 35). Airway inflammation was also significantly increased after

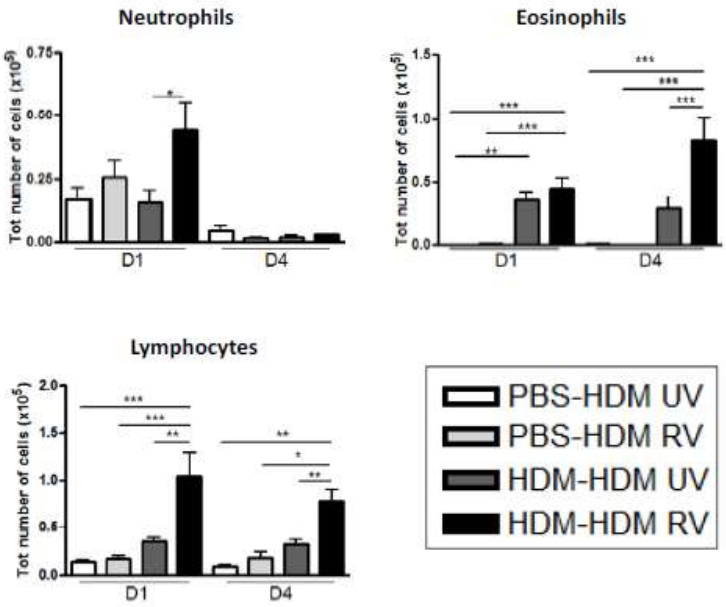

Figure 36 - Cellular inflammatory responses in the HDM rhinovirus-induced asthma exacerbation model. Mice were sensitized i.t. with HDM extract or PBS control and challenged i.n. with HDM and either RV1B or UV-RV1B control. At 24hrs and 4 days after infection bronchoalveolar lavage was performed and cell populations were enumerated by cytospin assay. ***p<0.001, **p<0.01, *p<0.05

exposure to the combined stimuli (Fig 36).

Looking into the mechanisms of antiviral responses, we used IL15AR and IFNAR knock-out mice to demonstrate that type-I IFN signaling is required for the production of IL-15, which in turn drives IFN-γ immune responses to RV. Blocking experiments show that type-I IFN signaling limits airway inflammation by reducing viral load.

Impaired production of the anti-inflammatory cytokine IL-10 has been observed in RV induced asthma exacerbations. To study the functional importance of IL-10 deficiency in the perpetuation of inflammation in asthma, we employed IL10 deficient (*IL-10*$^{-/-}$) mice. We challenged naïve mice with RV1b and followed the development of inflammation by assessing various parameters including leukocytic cell infiltration in the bronchoalveolar lavage fluid (BALF) and lung, and T cell cytokine production in the draining lymph nodes. We found that primary RV1b infection induced more severe inflammation in *IL-10*$^{-/-}$ mice compared to wild type mice, characterized by higher leukocyte and neutrophil counts in the BAL (Fig.37). However, in the OVA model IL10 deficient mice presented less inflammation and reduced Th2 responses. Possibly therefore, during allergic airway inflammation IL10 plays a detrimental role by sustaining Th2 responses.

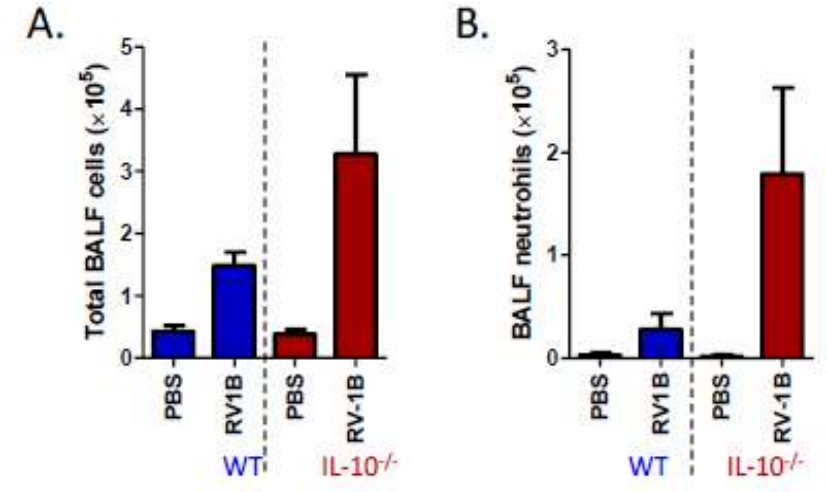

Figure 37 - Primary RV1b infection induced inflammation in wild type and *IL-10*$^{-/-}$ mice. Compared to wild type mice, characterized by higher leukocyte (A) and neutrophil (B) counts in the bronchoalveolar lavage fluid (BALF).

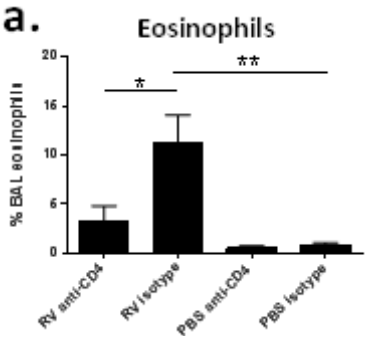

Figure 38

Furthermore, in mice lacking the Th1 master transcription factor Tbet, responses to RV display a Th2/Th17 mixed phenotype and eosinophilic cellular inflammation, with no effect on T-reg cells. This inflammatory response is T-cell dependent, as shown by CD4 cell depletion (Fig.38).

This further stimulated interest on the role of IL17 in RV infection; mouse and human studies suggest a possible pro-inflammatory role, enhancing neutrophil inflammation. We have identified a complicated interaction between IL17 and RV infection in mice and airway epithelial cells. An important gene necessary for virus clearance, OAS1, could not be upregulated in T-cells without IL17A. In addition IL17 is capable of downregulating LDLR, which is the receptor of minor type RVs, therefore prohibiting viral entry.Furthermore, RV is able to dowregulate IL17 production, possibly as a viral defense mechanism (Fig.39 & 40)

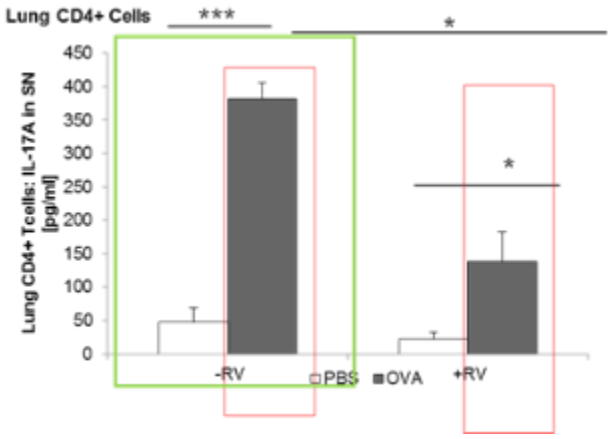

Figure 39 - Increased IL-17A production by CD4+ T cells isolated from the lung of asthmatic mice (OVA) as compared to those isolated from wild type mice. In vitro RV infection of these cells led to a strong reduction in IL-17A release.

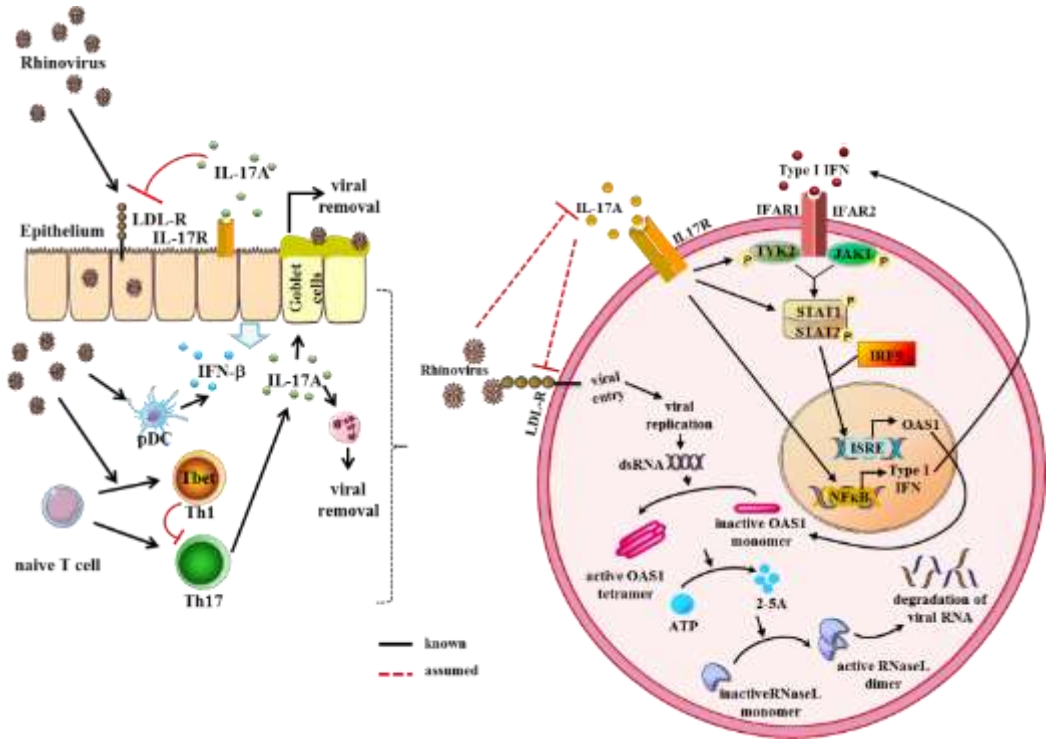

Figure 40. IL 17A mediated regulation of RV-induced inflammation
Upon infection, Type I IFNs bind to the IFNα receptor (IFNAR1 & IFNAR2) which initiates the signal transduction via JAK1 and TYK2. In the nucleus, OAS1 is transcribed upon induction of IFN-stimulated response elements (ISREs). In the cell cytoplasm OAS1 protein accumulates as an inactive monomer. Upon binding of double-stranded RNA (dsRNA), the enzyme oligomerizes to form a catalytic active tetramer. This active form synthesizes 2-5A using ATP as substrate. 2-5A binds to inactive RNaseL and triggers the dimerization of monomers. RNaseL then degrades RNA of viral and also cellular origin, leading to the inhibition of viral propagation. We suggest that IL 17A induces IFN-b and OAS1 and inhibits LDL-R expression, whereas in turn, RV suppresses IL 17A.

Persistence of asthma in preschool children

A preschool-to-school age paediatric cohort has been recruited from 5 centres around Europe, comprising of 169 children with asthma, followed up prospectively to evaluate the number and type of infections among other factors that may predict disease persistence. A 2-year follow-up period was completed using a telemedicine platform. Materials have been collected and a number of outcomes have been measured, including viral and bacterial pathogens, antibody responses, cytokine responses, and vitamin D, described in previous sections.

When compared with children of the same age, children with asthma were more exposed to tobacco smoke and molds, while they watched more hours of television. They also suffered significantly more from other allergic diseases, such as allergic rhinitis, atopic dermatitis, food allergy, insect sting allergy. They were also significantly more atopic (57% vs 21%) and had a family history of allergy and asthma. In addition, children with asthma reported significantly more respiratory infections of longer duration in the year before inclusion. For the majority a virus infection was the major trigger for symptoms. Mixed phenotypes were also frequent. There were no differences between children who had reported a virus-induced phenotype from those with an allergen-induced phenotype. At this young age, lung function was not notably affected and there were no significant differences with the controls.

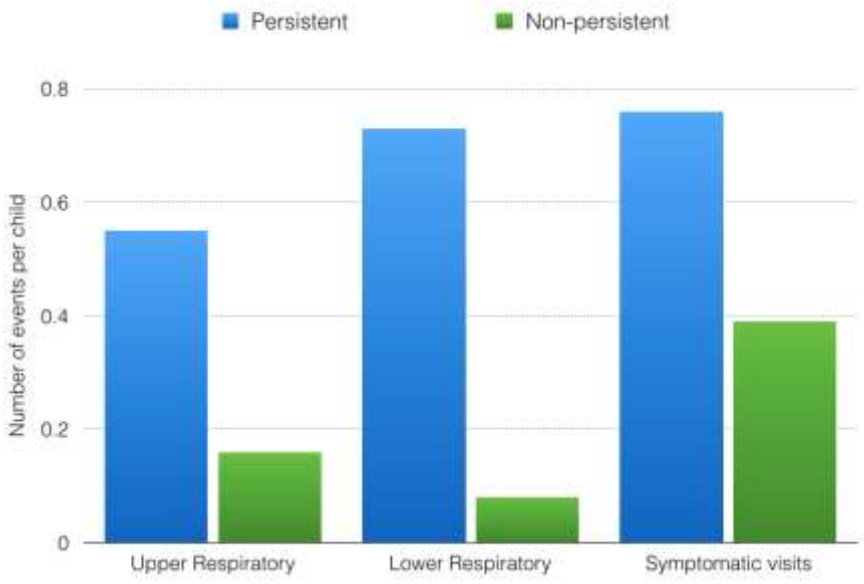

Figure 41 – Number of upper or lower respiratory infections as well as total symptomatic visits in children whose asthma persisted after 2 years (blue) and those who did not (green). Infections are strongly associated with persistence.

At the end of the observation period, data were available from 135 children, out of which 86 (63%) continued to have current asthma symptoms. The severity of asthma at recruitment, as well as lung function, were able to predict asthma persistence. Persistence was not affected by atopy, judged by skin prick tests.

Both upper and lower respiratory infections were associated with asthma persistence (Fig.41). The main hypothesis of PreDicta was therefore confirmed. The data produced by PreDicta will continue to be analysed in a systems medicine approach in order to identify further associations between persistence of asthma and the type, duration, severity and other characteristics of infections, as well as the innate and adaptive immunological responses.

Impact and dissemination activities

Respiratory diseases associated with allergy such as asthma and rhinitis constitute a major and continuously growing public health concern for Europe and globally, often referred to as 'the epidemic of the 21st century'. This has a profound impact in the daily quality of life of a very large proportion of Europeans and a very high cost to the European healthcare authorities and to the European economy in general.

PreDicta has evaluated the effect of infections on the persistence of such respiratory allergic diseases. This hypothesis was tested in human cohorts and mouse models and its validity and possible mechanisms have been thoroughly investigated. We have increased our knowledge on the molecular mechanisms of suboptimal innate immunity in epithelial cells in respiratory allergy. Furthermore, we have moved forward towards understanding in depth the effects of the innate immune system, affected by viral infection, on T-cell tolerance. We are able to explore inflammation resolution through relevant lipid mediators. New mouse models and platform technologies will boost European research and drug development on respiratory allergies.

The most ambitious aim of PreDicta was to establish diagnostic and therapeutic strategies to predict and if possible prevent respiratory allergy persistence. Towards this end, a diagnostic chip, able to recognize antibodies against RVs has being established. In addition, DNAZymes against RV have being developed and evaluated in vitro.

These results have improved the current understanding of asthma and rhinitis and will contribute towards the development of prevention programs. Accurate prediction of the predisposing risk factors for the persistence of respiratory allergies including asthma and rhinitis may have important socioeconomic benefits. New generation treatments using the latest targeted technologies (DNAzyme silencing) to interfere more effectively with the disease process by targeting causative agents rather than symptoms, can have groundbreaking impact, ensuring that discoveries benefit patients and very importantly children. Bringing down hospitalization costs as a consequence of earlier detection of the disease and development of new tools for monitoring disease initiation, progression, severity and treatment is an additional benefit.

Overall, PreDicta has advanced science in the field of respiratory allergies, and made bold steps towards the development of novel diagnostic and therapeutic interventions, strengthening the competitiveness of European research, boosting the innovative capacity of European health-related industries and businesses, and revealing ways for reducing health care costs, ultimately benefiting patients and the society as a whole.

Major project's outputs can be summarized as follows:

- Novel data on risk factors, pathogens involved and mechanisms of respiratory allergies.
- New mouse model and platform technologies for boosting European research and drug development on respiratory allergies.
- PreDicta's RV chip to identify the most relevant and clinically important RV strains involved in exacerbations of respiratory diseases and their long term effects
- Characterization of DNAzymes that cleave RV and may thus become an effective anti-RV intervention

During the course of the project, effective dissemination was key to communication of PreDicta's findings to scientists, physicians, health care organizations and policy makers, and the wider public, and increased the exploitation potential of the project:

- **Scientific publications**

To date (May 2016), 66 scientific publications were published in peer-reviewed journals and at least 10 other ones are in preparation.

D.O.I.	Title	Author(s)	Title of the periodical or the series	Date of publication
10.1016/j.jaci.2015.12.1344	Antisense molecules: A new class of drugs	Potaczek DP et al.	Journal of Allergy and Clinical Immunology	01/05/2016
10.1111/all.12931	Human Rhinoviruses Enter and Induce Proliferation of B Lymphocytes	Aab A et al.	Allergy: European Journal of Allergy and Clinical Immunology	01/05/2016
10.1111/all.12934	Viruses and bacteria in Th2 biased allergic airway disease	Lan F et al.	Allergy: European Journal of Allergy and Clinical Immunology	01/05/2016
10.1016/j.jaci.2015.11.047	Increased expression of nuclear factor of activated T cells 1 drives IL-9–mediated allergic asthma	Koch S et al.	Journal of Allergy and Clinical Immunology	01/03/2016
10.1016/j.jaci.2015.12.1300	International Consensus on Allergen Immunotherapy II: Mechanisms, standardization, and pharmacoeconomics	Jutel M et al.	Journal of Allergy and Clinical Immunology	01/02/2016
10.1016/j.iac.2015.08.003	Mechanisms of Aeroallergen Immunotherapy: Subcutaneous Immunotherapy and Sublingual Immunotherapy	Ozdemir C et al.	Immunology and Allergy Clinics of North America	01/02/2016
10.1111/all.12809	Barrier function of the nasal mucosa in health and type-2 biased airway diseases	Zhang N et al.	Allergy: European Journal of Allergy and Clinical Immunology	13/01/2016
10.1038/mi.2015.130	Rhinovirus inhibits IL-17A and the downstream immune responses in allergic asthma	Graser A et al.	Mucosal Immunology	06/01/2016
10.4193/Rhin15.213	Th2 biased upper airway inflammation is associated with an impaired response to viral infection with Herpes simplex virus 1.	Lan F et al.	Rhinology	23/12/2015
10.1097/IN.0000000000000872	Associations Between Viral and Bacterial Potential Pathogens in the Nasopharynx of Children With and Without Respiratory Symptoms	Skevaki CL et Al.	Lippincott Williams and Wilkins	04/08/2015
10.1016/j.jcf.2015.10.013	Interferon response of the cystic fibrosis bronchial epithelium to major and minor group rhinovirus infection	Schögler A et al.	Journal of Cystic Fibrosis	21/11/2015
10.1016/j.jaci.2015.05.006	CpG-DNA enhances the tight junction integrity of the bronchial epithelial cell barrier	Kubo T et al.	Journal of Allergy and Clinical Immunology	01/11/2015
10.1371/journal.pone.0136068	Association of Mucosal Organisms with Patterns of Inflammation in Chronic Rhinosinusitis.	Chalermwatanachai T et al.	PLoS One	14/08/2015
10.1016/j.jaci.2015.02.024	Association between respiratory infections in early life and later asthma is independent of virus type	Bønnelykke K et al.	Journal of Allergy and Clinical Immunology	01/07/2015
10.1016/j.jaci.2014.12.1866	T-cell regulation during viral and nonviral asthma exacerbations	Wegrzyn AS et al.	Journal of Allergy and Clinical Immunology	01/07/2015
10.1159/000430441	Infection with Rhinovirus Facilitates Allergen Penetration Across a Respiratory Epithelial Cell Layer	Gangl K et al.	International Archives of Allergy and Immunology	02/06/2015
10.1586/1744666X.2015.1035649	Contributing factors to the development of childhood asthma: working toward risk minimization	Guibas GV et al.	Expert Review of Clinical Immunology	01/06/2015
10.1371/journal.pone.0128564	Differential Expression and Release of Activin A and Follistatin in Chronic Rhinosinusitis with and without Nasal Polyps.	Yang Y et al.	PLoS One	01/06/2015
10.4049/jimmunol.1401	CCL7 and IRF-7 Mediate Hallmark Inflammatory and IFN Responses following Rhinovirus 1B Infection	Girkin J et al.	American Journal of Immunology	15/05/2015

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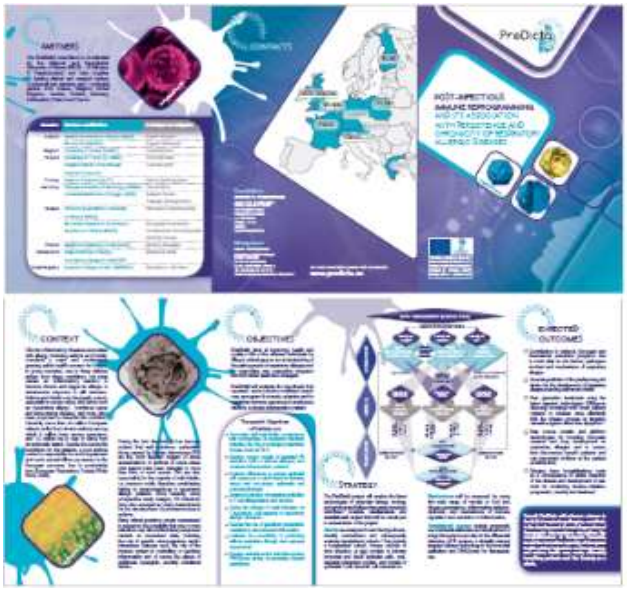
10.1093/cid/civ062	IL-18 is associated with protection against rhinovirus-induced colds and asthma exacerbations	D. J. Jackson et al.	Clinical Infectious Diseases	15/05/2015
10.1016/j.coviro.2015.03.004	Challenges in developing a cross-serotype rhinovirus vaccine	Glanville N et al.	Current Opinion in Virology	01/04/2015
10.1126/scitranslmed.aaa7390	Advances in allergen immunotherapy: Aiming for complete tolerance to allergens	C. A. Akdis et al.	Science Translational Medicine	25/03/2015
10.1016/j.jaci.2014.11.039	Increased nuclear suppressor of cytokine signaling 1 in asthmatic bronchial epithelium suppresses rhinovirus induction of innate interferons	Gielen V et al.	Journal of Allergy and Clinical Immunology	01/01/2015
10.1016/j.jaci.2014.12.1866	T-cell regulation during viral and nonviral asthma exacerbations	Wegrzyn AS et al.	Journal of Allergy and Clinical Immunology	01/01/2015
10.1164/rccm.201406-1039OC	IL-33–Dependent Type 2 Inflammation during Rhinovirus-induced Asthma Exacerbations In Vivo	D. J. Jackson et al.	American Journal of Respiratory and Critical Care Medicine	15/12/2014
10.1172/JCI78891	Mechanisms of immune tolerance to allergens: role of IL-10 and Tregs	Akdis CA et al.	Journal of Clinical Investigation	03/11/2014
10.1378/chest.14-0049	Scientific Foundations of Allergen-Specific Immunotherapy for Allergic Disease	Soyka MB et al.	Chest	01/11/2014
10.1126/scitranslmed.3010273	IL-25: The Missing Link Between Allergy, Viral Infection, and Asthma?	Andreakos E et al.	Science Translational Medicine	01/10/2014
10.1038/mi.2014.2	IL-15 complexes induce NK- and T-cell responses independent of type I IFN signaling during rhinovirus infection	Jayaraman A et al.	Mucosal Immunology	01/09/2014
10.1038/srep05865	Role of Tyk-2 in Th9 and Th17 cells in allergic asthma	Übel C et al.	Scientific Reports	11/08/2014
10.1016/j.coph.2014.07.003	Modulation of immune responses by immunotherapy in allergic diseases.	Cavkaytar O et al.	Current Opinion in Pharmacology	05/08/2014
10.1183/09031936.00179813	SFTA3, a novel protein of the lung: three-dimensional structure, characterisation and immune activation	Schicht M et al.	European Respiratory Journal	01/08/2014
10.1016/S2213-2600(14)70107-9	The role of macrolides in asthma: current evidence and future directions	Wong EH et al.	The Lancet Respiratory Medicine	01/08/2014
10.1183/09031936.00129513	Infantile growth velocity and later asthma/wheeze: GENESIS and the Healthy Growth Study	Guibas GV et al.	European Respiratory Journal	01/06/2014
10.1111/all.12329	Inhaled dsRNA and rhinovirus evoke neutrophilic exacerbation and lung expression of thymic stromal lymphopoietin in allergic mice with established experimental asthma	Mahmutovic-Persson I et al.	Allergy: European Journal of Allergy and Clinical Immunology	04/03/2014
10.1016/j.jaci.2013.12.1088	Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens	Akdis M et al.	Journal of Allergy and Clinical Immunology	04/03/2014
10.1136/thoraxjnl-2012-202909	Rhinovirus-induced interferon production is not deficient in well controlled asthma	Sykes A et al.	Thorax	01/03/2014
10.1016/j.jaci.2013.12.1032	The expression of cannabinoid receptor 1 is significantly increased in atopic patients	Martín-Fontecha M et al.	Journal of Allergy and Clinical Immunology	01/03/2014
10.1007/s11882-013-0413-5	The Common Cold: Potential for Future Prevention or Cure	Passioti M et al.	Current Allergy and Asthma Reports	01/02/2014

10.1016/j.jaci.2013.10.014	Human bocavirus 1 may suppress rhinovirus-associated immune response in wheezing children	Lukkarinen H et al.	Journal of Allergy and Clinical Immunology	01/01/2014
10.1016/j.jaci.2013.09.049	The activating protein 1 transcription factor basic leucine zipper transcription factor, ATF-like (BATF), regulates lymphocyte- and mast cell–driven immune responses in the setting of allergic asthma	Übel C et al.	Journal of Allergy and Clinical Immunology	01/01/2014
10.2500/ajra.2013.27.3956	Physical exercise increases nasal patency in asthmatic and atopic pre-school children	Haavisto LE et al.	American Journal of Rhinology and Allergy	01/11/2013
10.2500/ajra.2013.27.3922	Inflammatory patterns in upper airway disease in the same geographical area may change over time	Katotomichelakis M et al.	American Journal of Rhinology and Allergy	27/09/2013
10.1371/journal.ppat.1003669	Cross-Serotype Immunity Induced by Immunization with a Conserved Rhinovirus Capsid Protein	Glanville N.	PLoS Pathogens	26/09/2013
10.1111/cea.12152	Conception via in vitro fertilization and delivery by Caesarean section are associated with paediatric asthma incidence	Guibas GV et al.	Clinical and Experimental Allergy	01/09/2013
10.1007/s00705-013-1797-1	Entry of human rhinovirus 89 via ICAM-1 into HeLa epithelial cells is inhibited by actin skeleton disruption and by bafilomycin	Pfanzagl B et al.	Archives of Virology	04/08/2013
10.1371/journal.ppat.1003520	An anti-human ICAM-1 antibody inhibits rhinovirus-induced exacerbations of lung inflammation	Traub S et al.	PLoS Pathogens	01/08/2013
10.1038/mi.2012.118	Impaired innate interferon induction in severe therapy resistant atopic asthmatic children	Edwards MR et al.	Mucosal Immunology	01/06/2013
10.1002/emmm.201201891	Toll-like receptor 7 stimulates production of specialized pro-resolving lipid mediators and promotes resolution of airway inflammation	Koltsida O et al.	EMBO Molecular Medicine	01/05/2013
10.1038/srep01754	IL-6 activated integrated BATF/IRF4 functions in lymphocytes are T-bet-independent and reversed by subcutaneous immunotherapy	Koch S et al.	Scientific Reports	30/04/2013
10.3851/IMP2578	The application of prophylactic antibodies for rhinovirus infections	Privolizzi R et al.	Antiviral Chemistry and Chemotherapy	19/04/2013
10.1016/S0140-6736(12)62202-8	Microbes and mucosal immune responses in asthma	Hansel TT et al.	Lancet, The	09/03/2013
10.1016/j.jaci.2012.10.051	Triggering of specific Toll-like receptors and proinflammatory cytokines breaks allergen-specific T-cell tolerance in human tonsils and peripheral blood	Kücüksezer UC et al.	Journal of Allergy and Clinical Immunology	05/03/2013
10.1038/mi.2013.3	γδT cells suppress inflammation and disease during rhinovirus-induced asthma exacerbations	Glanville N et al.	Mucosal Immunology	06/02/2013
10.1002/emmm.201202032	Asthma exacerbations: a molecular dichotomy between antiviral and pro-inflammatory responses revealed	Andreakos E	EMBO Molecular Medicine	01/12/2012
10.1002/emmm.201201650	Defining critical roles for NF-κB p65 and type I interferon in innate immunity to rhinovirus	Bartlett NW et al.	EMBO Molecular Medicine	01/12/2012
10.1016/j.jaci.2013.06.010	Forkhead box protein 3 in human nasal polyp regulatory T cells is regulated by the protein suppressor of cytokine signaling 3	Lan F et al.	Journal of Allergy and Clinical Immunology	01/12/2012
10.1016/j.antiviral.2012.06.006	Rhinovirus infections and immunisation induce cross-serotype reactive antibodies to VP1	McLean GR et al.	Antiviral Research	01/09/2012
10.1371/journal.pone.0039875	Herpes simplex virus type 1infection facilitates invasion of Staphylococcus aureus into the nasal mucosa and nasalpolyp tissues	Wang X et al.	Plos One	30/06/2012
10.1016/j.jaci.2012.03.0	Rhinovirus 16–induced IFN-α and IFN-β are deficient in bronchoalveolar lavage cells in asthmatic patients	Sykes A et al.	Journal of Allergy and Clinical Immunology	01/06/2012

44				
10.1371/journal.pone.0044557	The Genomic Signature of Human Rhinovirus A, B and C.	Megremis S et al.	PLoS ONE	25/04/2012
10.1096/fj.11-193557	Misdirected antibody responses against an N-terminal epitope on human rhinovirus VP1 as explanation for recurrent RV infections	Niespodziana K et al.	The FASEB Journal	01/03/2012
10.1016/j.jaci.2011.09.031	Induction and maintenance of allergen-specific FOXP3+ Treg cells in human tonsils as potential first-line organs of oral tolerance	Palomares O et al.	J Allergy Clin Immunol	15/02/2012
10.1016/j.jaci.2011.12.1002	Inhibition of angiogenesis by IL-32: Possible role in asthma	Meyer N et al.	J Allergy Clin Immunol	15/02/2012
10.1002/emmm.201100142	IL-28A (IFN-λ2) modulates lung DC function to promote Th1 immune skewing and suppress allergic airway disease	Koltsida O et al.	EMBO Mol Med	03/06/2011
N/A	Od GA2LEN'a do MeDALL'u – alergologia w europejskich ramowych programach badawczych.	Kowalski ML	Biuletyn Informacyjny Uniwersytetu Medycznego w Łodzi	15/01/2011

- **Project presentation leaflet and Newsletters (all types of audience)**



A leaflet describing the project's objectives and expected outcomes was printed and distributed by the partners when attending scientific or public events.

Six public newsletters describing the project's progress were posted on the project website and a paper version was distributed during scientific congresses such as EAACI annual meetings:

-Mars 2011
-Oct 2011
-January 2012
-May 2012
-May 2014
-January 2015
-June 2015

- **Project Presentation video (all types of audience)**

A presentation video describing the project's objectives was posted on YOUTUBE and the project's website from the first months of PreDicta.

- **Participation of partners to public events (general public)**

PreDicta project was presented in public events such as the « Europe day » in Belgium, where the Upper Airways Research Laboratory of Prof. Bachert (UGENT) hold a booth. Visitors could undergo skin prick tests to determine whether they had some kind of allergy or get an idea about their lung function with a peak flow meter. The enthusiasm was big and more than 120 people were tested. The different PreDicta workpackages were explained to the visitors, most of which were surprised to discover the link between infection and allergy.

- **Participation to large international congresses (all types of audience)**

PreDicta partners all participated in national or international events/congresses where they presented their results obtain within the framework of PreDicta. The project's achievements were also recurrently presented during the European Academy of Allergy and Clinical Immunology annual Congresses (between 7000 and 9000 delegates from >100 countries) through posters, oral presentations and dedicated symposia, including June 2012 Geneva, June 2013 Milan, June 2014 Copenhagen, June 2015 Barcelona

A Symposium specifically dedicated to PreDicta was organised during the 2015 EAACI meeting: "*PreDicta: Tackling the infectious triggers of allergy*".

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PreDicta Consortium, Concluding Meeting, Athens 21 March 2016

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Hospital District of Southwest Finland (Turku UH)	Varsinais-Suomen sairaanhoitopiirin kuntayhtymä Intermunicipal Hospital District of Southwest Finland	Tuomas Jartti