Publishable Summary

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. 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. In addition to the consequences on the quality of life of patients, poor asthma control is responsible for work impairment and costs several billion per annum to the European economy due to productivity loss. The burden of allergic rhinitis and rhinosinusitis may be even higher.

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 (attacks) of asthma; in some cases viral agents have been detected in more than 90% of such events. 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.

Many critical questions remain unanswered in respect to the possibility that one or more infections may drive inflammatory responses towards an unresolved state, including the role of specific microorganisms and/or interactions between such, the role of the immune system in controlling or resolving inflammation and of course the effects of additional, synergistic, possibly subclinical factors.

Aims and hypothesis

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

Strategy and Objectives

PreDicta followed three interconnected work-flows: **models**, **mechanisms** and **translational output,** developed in several tasks within the project:

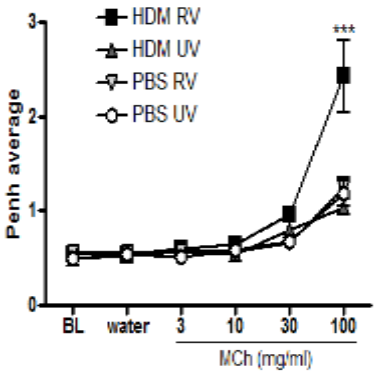
In the above context, the following specific objectives have been set:

- 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

Achievements

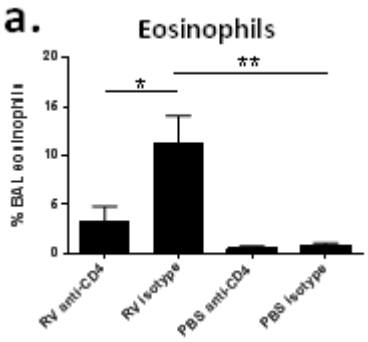
PreDicta has made remarkable progress towards understanding mechanisms of respiratory allergy persistence, setting up cohorts and models and achieving important milestones in translational outputs that will have added value and contribute towards its legacy. Over 65 publications in high level journals have documented PreDicta findings so far, while many more are expected.

A preschool-to-school age paediatric cohort has been recruited from 5 centres around Europe, comprising of 169 children with asthma, followed up closely to evaluate the number and type of infections among other factors that may predict disease persistence. A telemedicine platform has been used in order to monitor events in real time. The 2-year follow-up period was completed for all participants by month 60. The cohort provided material for the viruses, bacteria, antibodies, lipid mediators, cytokine responses that are described below. An association between upper and lower respiratory infections and asthma persistence has been documented. A number of additional and systems medicine queries have been planned, based on the cohort database and biobank.



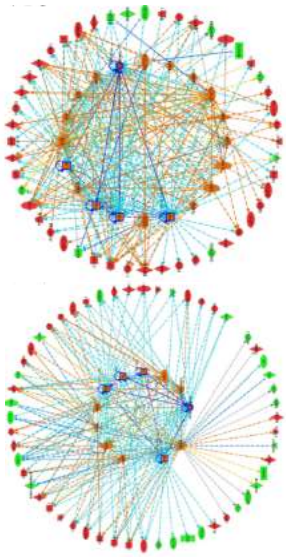
Mouse models designed to evaluate the effects of infection and interaction with allergic sensitization have been developed. An exacerbation model employing exposures to house dust mite (HDM) and subsequently rhinovirus (RV) demonstrated that increased hyperresponsiveness develops after the combination of exposures, rather than each factor alone (figure).
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

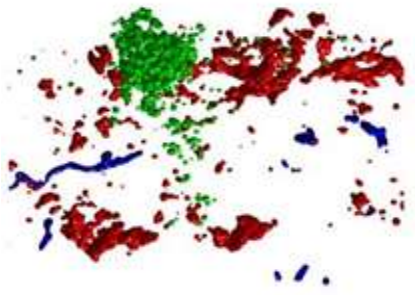
turn drives IFN-γ immune responses to RV. Blocking experiments show that type-I IFN signaling limited airway inflammation by reducing viral load.



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 (figure).
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. IL17 is required for induction of the antiviral gene OAS and was able to downregulate LDLR, the minor group RV receptor. Conversely RV is able to downregulate IL17 production.
In another set of experiments, a key regulatory cytokine, IL10, appears to be necessary for controlling RV-induced inflammation, but can be detrimental during allergic airway inflammation, by sustaining Th2 cell survival and cytokine production.

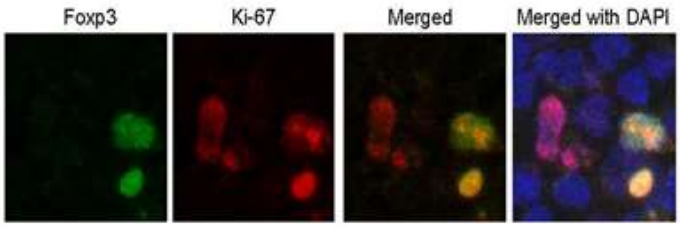
We have identified an important role for the epithelial derived pro-Th2 factors IL25 and IL33 in RV-induced asthma: such factors are produced by the respiratory epithelium in response to RV infection and can drive acute exacerbations, inducing innate lymphoid cell (ILC) production of IL5 and IL13. They correlate to exacerbation severity and asthmatic epithelium has an intrinsic capacity to overproduce them. Blocking their respective receptors attenuates virus-induced inflammation in the allergic background.
We used RNA-Seq to identify differentially expressed genes (DEG) between normal and asthmatic individuals in response to RV. It appears that reduced early transcriptional regulation takes place in asthma (figure: DEG expression and transcriptional regulation (inner circle) following RV infection of normal (upper) and asthmatic (lower) epithelial cells).
We have also demonstrated that SOCS-1, a nuclear factor controlling IFN is overexpressed after infection in cells from asthmatics.
IFN deficiency is present also in nasal epithelial cells of asthmatic subjects.



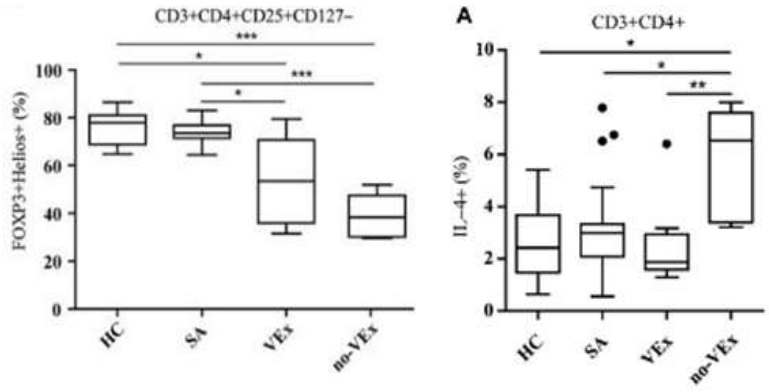


Combined exposure with RV16 and *St.* aureus compromises the epithelial layers (blue) and permits penetration of both microbes – 3D reconstruction of Confocal image. Green: SA, Red: RV

In an ex-vivo model of interactions between RV and Staphylococcus aureus (SA) infections in human nasal mucosa, pro-inflammatory cytokines were clearly increased after RV and/or SA infection; several of these mediators were common, however, the pattern of the combined response was clearly different from the individual ones.
Evaluation of RV16 and SA mucosal spread was performed by confocal microscopy. RV as a single infection led to very few infected cells of the outer epithelial barrier within 48h; 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, and SA was able to pass through the basement membrane and invade the mucosa.
Using tissue cultures from patients with nasal polyps it was shown that the response to a viral infection (with HSV) is deficient in IFN and IL-17 in the polyp tissue, while inflammatory response is increased in comparison to healthy mucosa. Furthermore, SA can induce directly TSLP and IL33 production in polyp tissue, resulting in an augmented Th2 response.

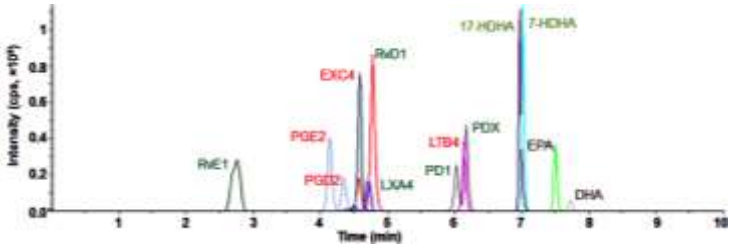


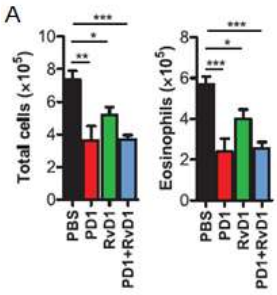
Ongoing studies are characterizing the effects of the innate immune response on tolerance. We have shown that triggering of Toll-like receptor (TLR)4 or TLR8 and the proinflammatory cytokines IL-1beta or IL-6 break allergen-specific T-cell tolerance in human tonsils and peripheral blood through a mechanism 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.
CD4+FOXP3+ Treg cells proliferate and co-localize with pDCs in-vivo in T-cell areas of tonsils (figure).



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 non-virus exacerbations, who also had significantly higher intracytoplasmic IL-4 in their CD3+CD4+ cells. Furthermore, the expression of IL-17 in CD3+CD4+ cells was upregulated in exacerbations.

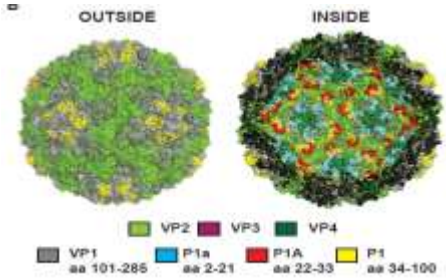
In order to study the active mechanisms of inflammation resolution, we developed and optimized LC-MS/MS methodologies that enable qualitative and quantitative determination of selected specialized pro-resolving lipid mediators (SPMs), including Lipoxin A4 (LXA4), Resolvin D1 (RvD1), Resolvin E1 (RvE1), Protectin D1 (PD1) and its stereoisomer 10S,17S-DiHDoHE (PDX). We have also expanded our panel towards pro-inflammatory molecules: Prostaglandin D2 (PGD2), Leukotriene B4 (LTB4) and Eoxin C4 (EXC4) (figure).

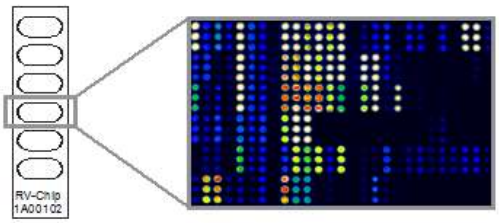




The developed methodologies were applied in human and mouse model settings, demonstrating increasing levels of SPMs paralleling inflammation resolution. Exogenous administration of SPMs, as well as stimulation through TLR7/8 binding, accelerated the resolution of inflammation (figure). The anti-inflammatory activity of dexamethasone was partly mediated through SPMs.

By studying antibody responses to RV, we observed that the major RV immune response was directed against an N-terminal non-neutralizing peptide in VP1 (P1A, red in the figure), which, paradoxically, is located inside the viral capsid. We suggested that the misdirection of antibody responses may serve as a possible explanation for the lack of protection against recurrent RV infections.

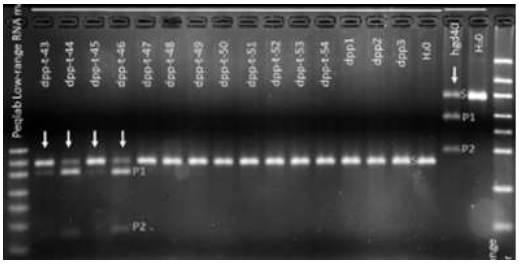


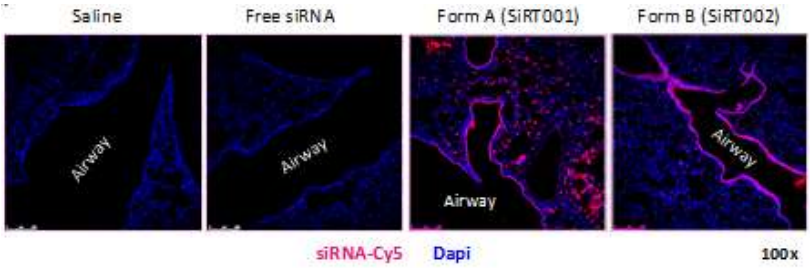


An array chip designed to characterize antibody responses to RV has been optimised and is able to discriminate RV group signatures. Use of this chip is expected to be a breakthrough in asthma exacerbation research and may prove key to the prediction of future outcomes. The humoral response to RV

was studied in a human experimental infection study as well as in a paediatric population; IgG1 antibody responses could be specifically identified and were highest in severe disease.
RV subtypes infecting children from our cohort were characterized; RV-C was predominant and at least one new serotype was found.

As an ambitious therapeutic strategy, we have developed and characterized DNAzymes that cleave RV and may thus become an effective anti-RV intervention. After considerable efforts, and among a large variety of putative regions, a number of candidate DNAzymes able to recognize RV have been identified and further developed for increased cleavage and wide strain coverage. These are being further developed towards preclinical evaluation.





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

Impact

PreDicta has evaluated the effect of infections on the persistence of respiratory allergic diseases. This hypothesis was tested in human cohorts and mouse models and its validity and possible mechanisms have been thoroughly investigated. Important advances in our understanding of molecular mechanisms of suboptimal epithelial responses, innate immunity and T-cell tolerance in respiratory allergy have been achieved. In addition, 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 expectations of PreDicta has been 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 been 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.

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