# Functional and clinical translation of asthma and allergy associated genetic variants in IL33 and IL1RL1

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# Chapter 10

Summary, general discussion and future perspectives

*"The road to find the best prescription for the patient as doctor and scientist: traveling from bench to bed and back in asthma/allergy"*

## Summary

In this thesis, I took a translational approach, connecting genetics of asthma and allergy to functional understanding and clinical application. Here, the focus was on two genetic loci frequently associated with asthma and allergy: *IL33* and *IL1RL1*. I put emphasis on the importance of defining specific asthma and allergy phenotypes in order to better connect genetics, functional consequences of the genetic polymorphisms and disease pathogenesis to clinical phenotypes. Knowledge of the mechanisms that drive the various disease phenotypes would thereby facilitate the design of targeted treatment strategies in specific subgroups of patients with asthma and allergy. Based on genetic associations, functional experiments and biomarker studies we were indeed able to define a specific asthma phenotype wherein the IL-33/IL-1RL1 pathway seems to be key.

**Chapter 1** introduces the research in this thesis by elaborating on the definitions of asthma and allergy, introducing the terms subphenotype and endotype. Furthermore, it touches on the different ways to unravel the genetic components of asthma and allergy: the methods to reveal the functional elements that drive the association of the genetic signals with disease; and the approaches to test whether and how this could be connected to pathophysiology and clinical application. With this, chapter 1 also specifies the genetic, functional and clinical evidence required to study the involvement of the IL-33/IL-1RL1 pathway in asthma and allergy.

**Chapter 2 and 3** summarize the genetic and functional evidence for the involvement of the IL-33/IL-1RL1 pathway in asthma and allergy at the time of starting my research in more detail. **Chapter 2** focuses on genetic evidence and potential functionality of asthma/allergy associated SNPs, by connecting asthma and allergy associated SNPs from literature in *IL33* and *IL1RL1* to functional elements in the disease-associated loci, including coding and noncoding effects. **Chapter 3** summarizes literature on the potential role of the IL-33 pathway in mast cell and basophil function in asthma and allergy; IL-33 being involved in the activation, degranulation and migration of these granulocytes relevant to development of chronic inflammation in asthma and allergy.

In the next part, this thesis moves towards translational and functional studies of genetic signals that associate with asthma and allergy. We define independent genetic signals in *IL33* and *IL1RL1* that associate with specific subphenotypes of asthma and allergy. In the research presented in **Chapter 4,** we focused on two independent genetic signals in *IL33* that contain potential eQTLs and particularly associate with an eosinophilic asthma phenotype. In the research described in **Chapter 5,** we identified four independent genetic signals in *IL1RL1*, of which three act as potential eQTLs and pQTLs for IL-1RL1 isoforms, and one encoding a (non-synonymous) change in four amino acids located in the signalling domain present in the transmembrane IL-1RL1 receptor, which we found to alter the IL-33 induced inflammatory response in an *in vitro* model. In **Chapter 6** we show that asthma-associated genetic signals in *IL1RL1* alter the gene expression pattern of Th2 cells in response to IL-33 stimulation in an *in vitro* model system using cells from asthma patients and healthy controls, a potential mechanism that may run via altered *IL1RL1* signalling and expression.

The third part of my thesis focuses on the clinical translation of the IL-33/IL-1RL1 pathway, with **Chapter 7** elaborating on the challenges that exist when measuring IL-33 protein in serum samples from asthma patients in clinical studies, highlighting problems with sensitivity, specificity and interfering factors when using current immuno-assay based detection methods. In **Chapter 8** therefore, the focus switches to IL-1RL1 levels in serum as a potential biomarker to predict the development of asthma phenotypes. In this research effort, we found that IL-1RL1 serum levels at age 2-3y did not contribute to the prediction of the broad phenotype of doctor's diagnosed asthma at age 5-6y, but did help to predict an eosinophilic asthma subphenotype in these preschool wheezing children, underlining the message that specific biological pathways contribute to specific disease phenotypes. In the research described in **Chapter 9,** we continued to study yet a different allergic phenotype namely that of food allergy. While IL-1RL1 serum levels did not predict the presence of food allergy, the *IL1RL1* genotype did, but only for specific food allergy phenotypes, again underlining the importance of the IL-33/IL-1RL1 pathway for specific subphenotypes of allergy. These results are summarized in figure 1 below.

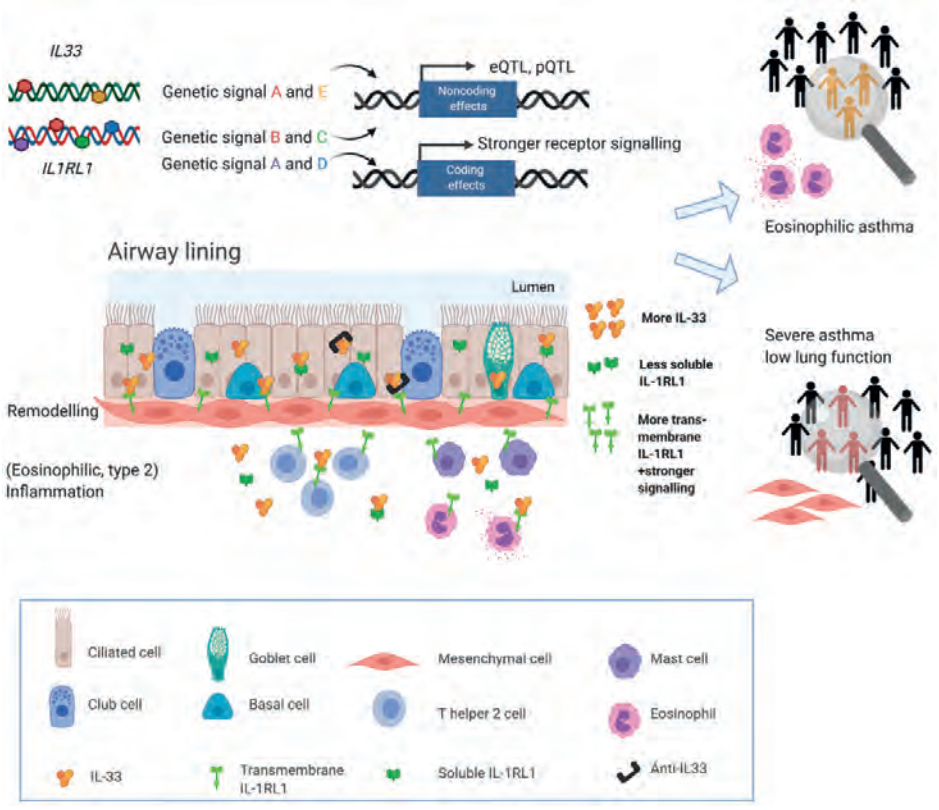


***Figure 1.***
In this thesis we described the distinct genetic signals in *IL33* and *IL1RL1* that specifically associate with an eosinophilic subtype of asthma (*IL33* signal A+E and *IL1RL1* signals A+D) and with severe asthma characterized by low lung function (*IL1RL1* signals B+C). The *IL33* risk alleles associated with higher levels of IL33 RNA in bronchial

brushes, as expression QTL (signal A and E). The *IL1RL1* risk alleles overall associated with lower levels of expression of soluble IL-1RL1 RNA and protein in bronchial epithelial brushes and cultured bronchial epithelium (signal A+D), but with higher levels of IL-1RL1 RNA in lung tissue (in signal B+C). Moreover, the asthma risk alleles of coding non-synonymous IL1RL1 SNPs (in signal D) were shown to associate with stronger signalling of the transmembrane IL-1RL1 receptor. Functionally, a contribution to eosinophilic, type 2 inflammation and airway remodelling might link these genetic signals to specific asthma subphenotypes. Targeted drugs directed at the IL-33/IL-1RL1 pathway may be useful drugs in asthma patients with these disease subtypes.

# Discussion

In this thesis, I have tested the overarching hypothesis that genetic variation at the *IL33* and *IL1RL1* loci and its gene products could have functional consequences for cells and tissues involved in the biological mechanisms underlying specific subphenotypes of asthma and allergy. Moreover, I hypothesized that the *IL33*/*IL1RL1* SNPs and IL-33/IL-1RL1 protein could have potential as biomarkers for the prediction of distinct asthma and allergy phenotypes and as targets for developing tailored treatment strategies in subgroups of patients with asthma and allergy.

### The IL-33/IL1RL1 pathway and specific asthma and allergy phenotypes

The appropriate definition of the disease phenotype is key to our ability to unravel the role of a molecular pathway in disease pathogenesis. This, however, is accompanied by many challenges. The first challenge is how to group phenotypes, given the high amount of overlap between various phenotypes regardless of what grouping factor(s) are used. For example, asthma phenotypes might be grouped based on factors such as severity of airway obstruction, age of onset, allergic component, type of immune cells in blood or sputum, but these do not define completely distinct phenotypes and patients may have different combinations of factors in their presentation of disease. The second challenge is the fluctuating nature of a phenotype over time (crystallization of disease during development, aging), the differences in presentation during an exacerbation versus stable phase of disease, and fluctuation of a phenotype in response to treatment. The third challenge is the availability of diagnostic tools that facilitate (and at the same time limit) the definition, selection and distinction of a phenotype. These all provide a genuine challenge for comparing phenotypes in scientific studies and clinical practice.

Asthma and allergy are often studied at the same time, as these have a large clinical overlap: around 60-65% of asthma patients have one or more comorbid or preceding history of allergic diseases, including chronic rhino-conjunctivitis, eczema, and less commonly food allergy (1,12,197). Moreover, asthma and allergic diseases share many genetic risk factors. (1) As suggested by the large overlap in clinical expression and genetic risk factors, asthma and allergic disorders may share common pathogenic mechanisms underlying the chronic inflammation observed in both conditions. However, allergies are only present in a subset of, but not in all asthma patients. This noticeably indicates that differences do exist, making a clearer definition of sub-phenotypes of asthma and allergy crucial to understand underlying

mechanisms of disease.

I think it is important to characterise patients based on a combination of (specific) clinical features, molecular characteristics (such as counts of specific immune cells, exhaled breath markers, cytokine signatures), and genetic risk factors to be able to stratify them as good as possible for treatments directed at molecular pathways (such as the IL-33/IL-1RL1 pathway). A hypothetical example: a person with severe (but still reversible) obstructive lung function, confirmed allergy against a specific allergen, early-onset of asthma, with elevated blood eosinophils and elevated levels of (Th) type 2 cytokines, carrying asthma-associated alleles in *IL33* (for example of rs992969) would potentially benefit from IL-33 directed treatment. In contrast, a person with mild obstructive lung function, non-allergic, adult-onset, not carrying asthma-associated alleles in either *IL33* or *IL1RL1,* but with high counts of blood eosinophils would perhaps be better off using different treatment strategies, for example drugs directed against IL-5. Obviously, this is a hypothetical example, and more study is required to better define the subgroup of patients as candidate for specific drugs combined with personalized clinical trials testing these hypotheses. Nevertheless, with this example I would like to suggest that future studies carefully define their included patients based on clinical, molecular and genetic factors to assess which combination of grouping factors could best predict a patient's response to specific treatments. Herein, the use of clinical features can guide the distinction of clinically relevant phenotypes, for example based on medical and family history, comorbidity, age of onset, lung function measures, determining an allergic component. A drawback of only characterising patients on clinical features is that clinical features may not necessarily represent distinct underlying biological mechanisms. Moreover, patient groups that exhibit similar clinical phenotypes may not share a common underlying pathobiology. (7,8) Therefore, grouping patients based on underlying biological features that could contribute to their symptoms, using specific cellular or molecular characteristics of the patient such as number and type of immune cells, exhaled breath markers or cytokines in sputum, bronchial washes or blood, or even transcriptome or epigenome of the cells in these compartments, could be a useful approach to unravel pathobiology of disease and find novel targets for intervention. Nevertheless, we are only starting to chart the causative biological mechanisms of disease, which is complicated by the redundancy of molecular pathways that lead to a specific symptom such as chronic airway inflammation in a single patient, as well as molecular markers may fluctuate over time as does a clinical phenotype. Therefore, using a combination of clinical factors linked to molecular features to group patients, the so called endotypes as introduced in chapter 1, would potentially be the way forward to distinguish patients for targeted treatment. Thus, in this thesis we used a combination of clinical features and molecular characteristics, and tested their use to further unravel pathogenic mechanisms of specific phenotypes, their applicability to clinical practice for eventual development of prediction models of disease and their potential for the development of personalized treatment strategies targeted at specific pathogenic pathways underlying a clinical phenotype.

In chapter 4 and 5 we used asthma as an umbrella term, and defined subgroups of asthma

patients based on clinical features and molecular characteristics (lung function, allergic component (atopy), age of onset, blood eosinophil levels, levels of specific IgE) in order to: 1) identify the specific asthma subphenotypes that *IL33*/*IL1RL1* SNPs may be involved in, and 2) to enable the translation of genetic signals in *IL1RL1*/*IL33* into a specific molecular process associated with specific asthma phenotypes. Additionally, in chapter 8 and 9, we started-off using general asthma/allergy definitions followed by studying specific sub-phenotypes, but then applied the IL-33/IL-1RL1 pathway as clinical prediction models: in chapter 8 we focused on wheezing children developing asthma phenotypes, while in chapter 9 we studied allergy either to any food or to specific food products.

The overarching message that could be derived from these 4 chapters is that the IL-33/IL-1RL1 pathway seems to be associated with specific disease phenotypes: the research presented in chapter 4, 5 and 8 indicates that this pathway associates with an asthma sub-phenotype characterized by presence of eosinophilic inflammation either in blood or the airways (asthma with high counts of blood eosinophils, asthma with high FeNO), and chapter 9 showing that the IL-33/IL-1RL1 pathway is not associated with food allergy in general, but did associate with clinical response to challenge with specific food allergies.

The finding that genetic variation both at the locus encoding the cytokine (*IL33*), and at the locus encoding the receptor (*IL1RL1*) is associated with a single asthma phenotype characterized by eosinophilia (chapter 4 and 5), strongly suggests that this would be the core phenotype the pathway could be involved in mechanistically. This is further supported by the finding that this association was found across 3 (different) asthma cohorts, indicating that the association of the *IL1RL1/IL33* pathway and an eosinophilic asthma phenotype could be generalized. Interestingly, we found further support for the association between the *IL33*/*IL1RL1* pathway and an eosinophilic predominant asthma phenotype when studying a paediatric cohort as presented in chapter 8. Although we did not find an association between *IL1RL1* SNPs and asthma development in this paediatric cohort, which might well be due to a lack of power for genetic association studies as this cohort had a limited number, we did observe that IL-1RL1 serum levels helped to predict the development of asthma characterized by high FeNO. High FeNO is considered a marker of eosinophilic airway inflammation. Nevertheless, blood eosinophils (used in chapter 4 and 5) do not perfectly correlate with eosinophilic airway inflammation, and FeNO is only considered a surrogate marker for eosinophilic airway inflammation. (263) This can be either seen as non-comparable phenotypes and coincidence of finding the *IL33*/*IL1RL1* pathway associated with both phenotypes, however, I interpret this as a sign of robustness of the association of the *IL33*/*IL1RL1* pathway with an eosinophilic predominant asthma phenotype even when applying different proxies of eosinophilic airway inflammation.

Further support for the association with an eosinophilic asthma phenotype can be found in other studies from literature, where SNPs in the IL-33/IL-1RL1 pathway associate with an eosinophilic predominant phenotype of the disease; moreover, SNPs at the *IL33* and *IL1RL1* loci were originally discovered as SNPs associated with level of blood eosinophils in an Icelandic general population cohort, but also - much more recently - in very large general

population cohorts. (15,17,20,25) Also, a recent study found that a (rare) loss of function mutation in *IL33* associated with lower blood eosinophil counts and to protect from asthma. (34) The functional genetic and mechanistic explanation for a contribution of the IL-33/IL-1RL1 pathway to chronic (airway) inflammation involving eosinophils will be discussed later in this chapter.

Furthermore, while we found that the *IL33* locus was associated with eosinophilic inflammation, it was not associated with lung function or severity of asthma (chapter 4). In contrast, genetic signals at the *IL1RL1* locus were associated with lung function or severity of asthma in addition to blood eosinophil counts (chapter 5). This discrepancy is an intriguing observation. Since these two loci were studied in the same cohorts using similar number of subphenotypes and SNPs at both loci had comparable allele frequencies, the observed difference in the association with these additional asthma phenotypes might not be derived from a technical bias or lack of power. Instead, this could represent a true difference in the subphenotypes that genetic variation at the cytokine locus versus the receptor locus are associated with. Potentially, this indicates a difference in the biological pathways that the cytokine and receptor are involved in functionally, possibly indicating a role in asthma inflammatory pathways (cytokine+receptor) versus remodelling/lung function (only the receptor), which will be discussed in more detail below.

## The IL-33/IL-1RL1 pathway and underlying mechanisms of asthma and allergy

Once we established the association of genetic variation at these two loci with specific phenotypes, we moved towards a functional genetics approach as previous studies had suggested functional effects of asthma and allergy associated SNPs in relation to functional genetic elements at *IL1RL1* and *IL33*. (212,278) To this end, we defined independent phenotype-associated genetic signals based on the LD structure at these loci. In addition, we looked for potential functional elements in the disease-associated genetic signals. Then, the selected signals were used in eQTL and pQTL studies to detect potential noncoding effects via regulation of gene expression (*chapter 4, 5, 8 and 9*). To model the effect of altered *IL33* gene expression we performed functional experimental studies, analysing the cell-autonomous effects of overexpression of *IL33* in primary bronchial epithelial cells and of increased exogenous IL-33 levels on cell signalling and cell behaviour in primary bronchial epithelial cells and Th2 cells (*chapter 4, 5, 6*). Moreover, coding effects of phenotype-associated signals in *IL1RL1* were analysed in functional experimental studies by specifically overexpressing the two main exon 11 haplotypes of *IL1RL1* (chapter 5). In these functional genetics approaches, we used cells and tissues relevant for asthma/allergy pathogenesis, including lung tissue, bronchial biopsy and brush samples, as well as primary human bronchial epithelial cells and Th2 cells.

From our functional genetics studies, we have learned the following five lessons, which we first summarize and then discuss in more detail below:

**First**, the *IL33* locus has two independent, genetic signals in *IL33* that are associated with

specific phenotypes. These two signals are located in expression-related functional elements in non-coding areas and are strong eQTLs. In contrast, the four independent genetic signals in *IL1RL1* that have an association with specific phenotypes, have both noncoding effects (eQTLs and pQTLs) that alter the expression levels of the two protein isoforms and coding effects that change signal transduction capacity of the transmembrane receptor.

**Second**, the overall direction of effect for the eQTLs in *IL33* and *IL1RL1* is an association with increased *IL33* expression and reduced expression of the soluble isoform of IL-1RL1, with only a few exceptions in case of *IL1RL1*. Overall, this seems to suggest an increased activity of the pathway to be associated with an increased risk of (eosinophilic) asthma.

**Third**, this increased activity of the IL-33/IL-1RL1 pathway seems to lead to diminished bronchial epithelial cell function (less viability and lower capacity of ROS scavenging) and to increased transcriptional activation of Th2 cells, the latter again especially in *IL1RL1* asthma-risk haplotype carriers.

**Fourth**, the genetic signals that associate with eosinophilic asthma and eosinophilia are eQTLs in bronchial epithelium (*IL33* signals A and E, *IL1RL1* signal A), while the genetic signals associated with lung function and severe asthma (*IL1RL1* only, see signals B, C) were identified as eQTL in lung tissue, but not in bronchial epithelium, indicating the possible involvement of other lung structural cells for the expression of the phenotype.

**Fifth**, the environment seems to influence the effect of genotype on gene expression for *IL1RL1* but not for *IL33*, as evident from HDM and RV16 induced eQTLs in epithelial cells only for *IL1RL1*.

Firstly, non-coding effects were identified among the disease associated genetic signals at both *IL33* and *IL1RL1*, while in addition coding effects existed for the *IL1RL1* locus. The genetic structure at the latter region showing more (genetic and functional) complexity. For *IL33* we find that the genetic signals that associated with asthma phenotypes contain potential functional expression-regulatory elements. We show the association of these genetic signals with *IL33* expression. As IL-33 is considered to function as an alarmin upon damage of (epithelial) cells (37,105,137,140,174,260,279), genetic variation that would lead to increased levels of IL-33 might well result in increased activation of downstream inflammatory pathways, which in turn could contribute to the susceptibility for chronic inflammatory diseases such as asthma. In such a scenario, damage of the airway epithelium would more readily lead to chronic airway inflammation in carriers of *IL33* haplotypes that we found associated with increased *IL33* expression (eQTL studies chapter 4). Interestingly, for the *IL1RL1* locus we found both (non-coding) eQTL effects as well as coding effects of asthma- and allergy- associated genetic variants. The regulation of IL-33 signalling through IL-1RL1 is tightly regulated, with IL-33 being released only during necrotic, not apoptotic cell death; the activity of IL-33 being highly sensitive to activating and inactivating proteases, as well as the presence of an antagonistic soluble isoform of the receptor and negative feedback loops at the receptor level. (137,140,174,211,232,260,279) The relative expression levels of the (decoy) soluble receptor and the transmembrane receptor capable of signal transduction would be crucial in finetuning the activation status of the IL-1RL1 pathway. Our data indicate

that the non-coding eQTLs at the *IL1RL1* locus mainly affect the soluble IL-1RL1 isoform, whereas there is far less variation in the expression level of the transmembrane receptor. In addition to variants regulation expression levels, coding effects were found influencing the activity of the transmembrane receptor. Genetic variation in exon 11 encoding the intracellular signalling domain (which is absent in the soluble isoform) results in amino-acid changes that influence the signalling capacity of the receptor (*chapter 5*).

Secondly, these non-coding and coding effects seem to lead to increased activity of the IL-33/IL-1RL1 pathway: higher expression of *IL33* was seen in risk alleles associated with increased blood eosinophil counts, and increased chance of asthma and eosinophilic asthma. But also an investigation of the *IL33* signal A SNP rs992969 in the food allergy cohort (chapter 9), showed that this allele was associated with higher chance of asthma and eczema in food allergic children. However, no association with food allergy was found. No *IL33* SNPs were available for a look-up in the paediatric asthma cohort of chapter 8. The positive direction of effect between disease-association and higher expression of *IL33* is in agreement with literature (18,238). For example, Luo et al. (238) studied publicly available GWAS and eQTL data focusing on airway epithelium, and found that asthma-risk alleles in *IL33* associated with increased *IL33* expression in airway epithelial samples. Moreover, Pividori et al. (18) found in the UK Biobank that *IL33* SNP rs992969 associated with childhood-onset asthma, and the risk allele associated with increased expression of *IL33* RNA in skin, blood and intestine. In our studies of *IL1RL1,* the direction of effect of different asthma associated signals was more complex: we found 4 independent phenotype associated signals (A-D) that were also *IL1RL1* eQTLs and/or pQTLs. For the genetic signals A and D, the alleles associated with a high count of blood eosinophils and with increased chance of asthma (see table 4 in chapter 5) were associated with lower levels of (soluble) IL-1RL1 either on RNA or protein level. Indeed, this is also seen in our other cohorts, such as the paediatric cohort in chapter 8 where the investigated SNP (rs1420101, representing signal D) was associated with lower levels of soluble IL-1RL1, and lower IL-1RL1 levels added to a predicted higher chance of developing eosinophilic (FeNO-positive) asthma. Moreover, other studies from literature also indicate that lower levels of soluble *IL1RL1* RNA or IL-1RL1 protein associated with asthma and eosinophilia risk alleles (27,239). In childhood asthma samples, as well as in adult asthma cohorts, both Savenije et al. and Dijk et al. found that asthma-risk alleles in *IL1RL1* associated with lower serum IL-1RL1a and with higher blood eosinophil counts (27,239). Indeed, although environmental and clinical factors are likely to explain some of the variation in soluble IL-1RL1 levels in serum, studies show that much of the variation in soluble IL-1RL1 levels is driven by genetic factors (31).

What we have not studied in this context, and would be relevant to do in future is to also compare the relative magnitude of change in expression as consequence of genetic variation for both *IL33* and *IL1RL1*, i.e. is soluble IL-1RL1 indeed decreased to a sufficiently low level to be biologically relevant to increase bio-availability of IL-33 to a level that will lead to enhanced inflammation?

For *IL1RL1* we found some exceptions in the direction of effect: in chapter 5 for the *IL1RL1*

signals B and C, associated with lung function and severe asthma respectively, the disease associated allele was associated with increased levels of both soluble and transmembrane *IL1RL1* encoding RNA. Apparently, in severe cases of asthma characterized by low lung function, a different disbalance of the IL-1RL1 soluble and transmembrane isoforms may be present; which was also observed during exacerbations in severe asthma patients (56,280). For example, Oshikiwa et al. (280) found an increase in serum IL-1RL1a during severe asthma exacerbation. Mechanistically, this could be a sign of uncontrolled feedback in severe inflammation, where IL-33 is exhaustively released, turning on expression of IL-1RL1, and potentially people carrying the risk-alleles in signal B and/or C being more prone to develop uncontrolled inflammation. The positive direction of effect of disease-risk and expression levels of the soluble receptor was also seen for the risk-alleles of *IL1RL1* that were studied in the context of specific food allergy (chapter 9): the *IL1RL1* alleles that associated with higher chance of peanut allergy associated with higher levels of soluble IL-1RL1 protein. Apparently, not only the severity of inflammation, but also the target organ (intestine) affects the direction of the disbalance of IL-1RL1.

Hence, the asthma-associated genetic variation at the *IL33* and *IL1RL1* loci can be summarized to result in non-coding (eQTL) effects for *IL33* (more expression for the susceptible allele, two discrete signals), and non-coding and coding effects for *IL1RL1* (four discrete signals). With soluble *IL1RL1* eQTLs (overall less expression for the susceptible allele) and non-synonymous coding SNPs for the transmembrane *IL1RL1* receptor (susceptible allele rendering a more potent receptor). Nevertheless, there were some exceptions in the direction of effects and we cannot exclude that also coding effects for *IL33* SNPs exist, as suggested by the rare loss off function variant recently found (34).

Thirdly, the overall increased activation/signalling via the transmembrane receptor IL-1RL1b had functional consequences for bronchial epithelial cells and Th2 cells. We found that overexpression of *IL33* altered the viability and ROS-scavenging capacity of bronchial epithelial cells (chapter 4). Moreover, the asthma-relevant Th2 cells showed altered (pro-inflammatory) gene expression patterns upon IL-33 stimulation including effects on IL-5 and IL-13 expression, with these effects being much more prominent in carriers of asthma-associated *IL1RL1* haplotypes. The genetic variation that we stratified for in the Th2 study might have direct functional effects via increased *IL1RL1* expression. Also, it may run via the non-synonymous changes in the amino acid sequence of the exon 11 part of the gene encoding the intracellular signalling domain of IL-1RL1b as we had seen in chapter 5 where we showed increased NF-κB signalling upon IL-33 treatment in cells overexpressing the *IL1RL1* exon 11 risk haplotype.

An interesting fourth observation was that the genetic signals associated with eosinophilic asthma and eosinophilia seems to be eQTLs in bronchial epithelium (*IL33* signals A and E, *IL1RL1* signal A), while the genetic signals associated with lung function and severe asthma (*IL1RL1* only, see signals B, C) were eQTL in lung tissue, but not in bronchial epithelium. This seems to indicate a difference in cell types that (mainly) express these genes and a difference in their relative level of expression in these cell types. Also, in the single-cell expression atlas of

the lung (182) it was observed that there is limited overlap in cells expressing *IL33* and *IL1RL1.* Therefore, any association of SNPs with gene expression levels of *IL33* and *IL1RL1* will have a divergence in cell-type dependency: *IL33* eQTL signals are driven by other cell types compared to *IL1RL1* eQTLs. Consequently, the presence of *IL1RL1* eQTLs in lung tissue associated with lung function and asthma severity indicate the presence of a specific cell type in lung parenchyma that contributes to these phenotypes and expresses *IL1RL1*. For example capillary endothelial cells in lung tissue express high levels of *IL1RL1*, (182) but their contribution to lung function parameters remains to be tested. While structural cells such as fibroblasts expressing *IL1RL1* could contribute to remodelling of lung tissue (281), the potential differential effect of *IL1RL1* genotypes in these cells also needs to be further investigated.

Thus, while our studies suggest that the cytokine encoded by *IL33* contributes to the initiation of an inflammatory response involving bronchial epithelium and immune cells such as eosinophils, the genetically encoded alterations in expression or function of the receptor might not be restricted to act in an inflammatory pathway. I recommend to perform cell type specific eQTL experiments using sources such as the single-cell expression atlas of the lung, to further unravel the role of different disease-associated genetic variants in distinct cell types, relevant at different stages of disease, and perhaps for development of specific disease phenotypes. With clinical application in mind, it would also be highly relevant to include comparison to easier accessible airway epithelial cells, such as nasal epithelium. Imkamp et al. (190) for example showed that these could have potential as proxy for bronchial epithelial cells.

In light of the role of the IL-33/IL-1RL1 pathway in several disease stages, a recent study in an experimental mouse model of asthma showed that anti-IL-33 treatment reduced the Th2 inflammatory response characterised by eosinophilia in the acute exacerbation phase, but had no benefit for airway smooth muscle remodelling in later phase of disease. (282) In contrast, another asthma mouse model study using an anti-IL-33 neutralizing antibody showed benefit for both inflammation and remodelling when treating mice with anti-IL-33. (283) However, these two models were using different experimental asthma models, the first studying a virus-induced (semi)acute asthma exacerbation model, whilst the second was an HDM-model of chronic airway remodelling after an inflammatory induction phase. Unfortunately, neither of these studies investigated anti-IL-1RL1 as therapeutic strategy in the context of lung tissue remodelling, and the main source of IL-33 in the mouse model is the type-2 alveolar epithelial cell, which in human lacks IL-33 expression (177), further limiting the translational impact of these models. In human studies, the IL-33/IL-1RL1 pathway has also been linked to airway remodelling in asthma, with one study suggesting IL-33 as cytokine to be an important mediator of airway wall thickening (253) studying 45 asthma patients and 40 controls, but the IL-1RL1 receptor was not studied. A second study suggested the receptor to be crucial in airway wall remodelling in severe steroid resistant paediatric asthma, as knocking-out the receptor in fibroblasts taken from these patients strongly reduced collagen synthesis (35). Whether IL-33 and the receptor would indeed have different roles in different phases of airway disease, or they act in synergy

would be interesting to study, as this potentially would mean different treatment strategies in different phases of the disease. Therefore, very relevant would be a study comparing 1) acute inflammation and (chronic) remodelling in the same asthma model, and 2) anti-IL-33 and anti-IL-1RL1 treatment in a direct comparison to see whether cytokine vs receptor have different roles in different phases/components of disease pathophysiology.

A fifth and last observation that we made in our functional studies was that triggers from the environment had a different effect on expression in asthma risk and asthma-protective haplotypes for *IL1RL1* (chapter 5) but not for *IL33* (chapter 4). In other words; we found evidence for inducible (or conditional) QTLs (iQTLs) for *IL1RL1* but not for *IL33*. In our studies we used the asthma-relevant triggers HDM and RV16. For *IL1RL1* HDM induced an increase in soluble IL-1RL1a protein levels unstratified for genotype, as well as in carriers of an asthma/eosinophilia protective allele (signal A and D, figure 5 of chapter 5), i.e.: more decoy receptor was present upon allergen exposure especially in patients carrying asthma protective haplotypes. For signal C, the severe asthma signal, this was the other way around: HDM induced an increase in the expression of the soluble IL-1RL1 protein in allele carriers at risk for developing severe asthma. Apparently, as for the baseline QTL effects, for signal C a different environment-genotype interaction exists. We did not observe any inducible eQTL or pQTL effects for RV16 for *IL1RL1* haplotypes. Interestingly, for *IL33*, although both asthma/eosinophilia risk genotypes and the environmental triggers HDM/RV16 induced *IL33* expression (RNA level, see chapter 4 figures E7-E9), exposure to HDM or RV16 had no additional effect on *IL33* expression above the genotype effect. I.e. no inducible QTLs existed here, in even other words; no gene-environment interaction was found for the studied *IL33* genotypes and studied environmental triggers.

The fact that we found iQTLs for the receptor, but not for the cytokine, in the same primary bronchial epithelial cell cultures, could potentially mean that these environmental triggers, particularly HDM, have an additional effect on risk of developing asthma on top of genetic effects for the receptor only. But of course this is just a first observation and should be studied in other cohorts before drawing too firm conclusions. A very relevant remark here is that the allergen-induced effects we found for *IL1RL1* were on protein level, whilst for *IL33* we were limited to the RNA level as the protein is hard to quantify (see also chapter 7 discussing the challenges that exist when measuring IL-33 protein). Whether or not allergen-induced pQTL effects exist for *IL33* requires further study dependent on the development of reliable IL-33 assays. Biologically, allergens might very likely affect IL-33 protein levels and activity, for example HDM is known to contain proteases that proteolytically could affect IL-33 as was studied in mice (284). Truncated forms of IL-33 may have different potency, may lack certain protease sites, and/or may lead to different cellular localization of the IL-33 protein in reach or out of reach of proteases. A relevant mechanistic future study would then be to 1) investigate the effects of HDM or other allergens on the presence of IL-33 protein isoforms and 2) studying the activity of these isoforms, linked to genetic variation at *IL33*.

Thus, our genetic association and functional studies suggest that genetic signals at both *IL33* and *IL1RL1* associate with eosinophilic predominant phenotypes via altered expression and

receptor signalling in bronchial epithelium, but that some *IL1RL1* signals associate with additional phenotypes potentially involving more structural changes. Herein the balance between IL-33 levels, and levels of the soluble and transmembrane IL-1RL1 seems to be crucial. Considering the above, I would hypothesize that for IL-33, relatively straightforward strategies that target the increased IL-33 levels (e.g. IL-33 blocking antibodies) would be beneficial in reducing chronic inflammation in carriers of specific *IL33* genotypes in several forms of (allergic) disease. Whilst for IL-1RL1, 'simple' blocking strategies of the receptor would require a more sophisticated approach, respecting the balance between the soluble and transmembrane form and such a treatment may have different effects for different severities and elements of disease, and for different target organs. However, evidence is emerging that also for IL-33 several isoforms exist on both RNA level, and on protein level upon post-translational modification of IL-33, conveying different activity (174,278,284,285), with some perhaps more relevant for disease pathogenesis than the other. A first study investigating RNA isoforms of *IL33* (278) suggests that the expression of exon 3 and 4 is related to type 2 inflammatory responses. The rare genetic variant in *IL33* that associates with lower eosinophil counts and protects from asthma, potentially acts via truncation of the last intron, predicted to result in a premature stop codon that leads to truncation of 66 amino acids of IL-33. (34) If certain SNPs are associated with isoforms that are predicted to encode forms of IL-33 protein that are directed intracellular, intranuclear or predicted to be secreted would potentially shed a different light on the role of *IL33* SNPs in the context of asthma-pathogenesis. With this it also seems that strategies targeting IL-33 may require more sophisticated approaches than anticipated now, directed at specific isoforms. Future studies to unravel the regulation and function of RNA and protein isoforms in the context of asthma and allergic disease are therefore urgently needed to intelligently guide development of targeted treatment.

### The IL-33/IL-1RL1 pathway in clinical prediction

As we found a potential functional role of *IL33* and *IL1RL1* SNPs in the molecular mechanisms underlying asthma and allergy, we next moved to clinical prediction: could *IL33* and *IL1RL1* SNPs and their protein expression levels be used as biomarkers?



Since we found a strong genetic signal (tagSNP rs992969) at the *IL33* locus associated with an eosinophilic predominant asthma phenotype and with *IL33* expression, we considered the option that IL-33 could be used as biomarker for asthma phenotypes. To this end we first aimed to determine IL-33 protein levels in serum of asthma patients to see whether systemic levels of this cytokine holds biomarker potential. In chapter 7 we described the challenge of reliably quantifying IL-33 protein in serum and concluded that the currently available immunoassays lacked sufficient sensitivity and specificity to enable the measurement of this cytokine in serum samples, especially due to the presence of interfering factors, such as soluble IL-1RL1 present in this biological challenging specimen. Thus, this field would strongly benefit from novel specific detection methods to quantify IL-33 protein, potentially including an IL-1RL1 capture step, or developing high affinity antibodies directed at IL-33

competing with soluble IL-1RL1, or antibodies able to detect an IL-33/IL-1RL1 complex. Non-antibody-based systems are limited, but could be promising once more developed, including bio-sensing platforms (286).

For IL-1RL1, we explored the use of *IL1RL1* SNPs as well as IL-1RL1a serum levels as biomarker for the prediction of asthma and allergy phenotypes in paediatric cohorts in chapter 8 and 9. Thus far, some mixed messages derive from literature regarding the use of *IL1RL1* SNPs in the prediction of asthma and wheezing phenotypes. For example, in a previous paediatric cohort (ALSPAC combined with a subset of PIAMA) *IL1RL1* SNPs were associated with several wheezing phenotypes, including persistent wheeze (28). However, a recent study of the larger PIAMA cohort, also including a different paediatric cohort as replication cohort (BAMSE) (287), concluded that asthma-associated SNPs compiled as genetic risk score did not add to the prediction of childhood asthma. These did not study specific subphenotypes, such as eosinophilic asthma while blood eosinophil counts were available for a subset of children. In our investigated paediatric cohort of preschool-age wheezing children (ADEM cohort, chapter 8) we only had one *IL1RL1* SNP available for predictive testing (rs1420101). This SNP was not associated with the development of asthma in wheezing preschool children, nor did rs1420101 add to the prediction of asthma on top of the already existing Asthma Prediction Index (API). Also when defining a subgroup of patients, the FeNO high (eosinophilic) asthma group, we did not find rs1420101 of predictive use. This could have technical explanations, as the ADEM cohort had a relatively limited sample size for genetic analyses (202 children, and the subdivided group even less). Or it might be that this particular SNP does not hold predictive value in paediatric wheezing/asthma cohorts; the latter may be plausible, as retrospectively this particular SNP did not associate with persistent wheeze and/or asthma in the ALSPAC or PIAMA.

Our biomarker study of IL-1RL1a in the ADEM cohort was better powered. Interestingly, serum IL-1RL1a had predictive value (on its own and in addition to the asthma prediction index), but only for the FeNO-high eosinophilic asthma phenotype.

In all these asthma cohorts, the studied *IL1RL1* SNPs were associated with serum IL-1RL1a levels, which implicates that *IL1RL1* SNPs could be valuable for prediction of the variation in serum IL-1RL1a even in smaller cohorts, but that this association does not necessarily translate into predictive value for asthma phenotypes in such cohorts.

When moving to food allergy (chapter 9), *IL1RL1* SNPs also associated with serum IL-1RL1a, but some SNPs did in addition have phenotypic predictive value, for food allergy against specific foods. This indicates that also in food allergy it is of interest to investigate specific disease phenotypes. Here, *IL1RL1* SNPs were associated with IgE sensitization against chicken egg and peanut. Only for peanut allergy *IL1RL1* SNPs were associated with the clinically (DBPCFC) proven food allergy. The direction of effect for the association with expression levels was also different for chicken egg and peanut: SNPs associated with chicken egg sensitization had a negative direction of effect on IL-1RL1a levels, the risk allele associating with lower levels of serum IL-1RL1a protein levels, while for peanut allergy the risk allele associated with higher levels of IL-1RL1a in serum. Whether this observation

could mean a different underlying mechanism leading from sensitization to clinical allergy between chicken egg and peanut foods requires further study.
Thus, the clinical prediction part of the thesis showed mixed evidence for *IL1RL1* SNPs and IL-1RL1 as potential biomarker for the prediction of asthma and allergy phenotypes in paediatric cohorts and we did not have the ability to explore *IL33* SNPs or IL-33 protein as predictors of disease. The clinical use of *IL1RL1* SNPs could potentially be in the detection of IL-1RL1a high vs low subjects, while levels may have more potential for prediction of asthma and allergy phenotypes. Herein, it seemed crucial to define specific phenotypes in both asthma and food allergy. Before we can conclusively state whether or not SNPs and expression levels of the IL-33/IL-1RL1 pathway could have predictive potential in asthma and allergy, carefully designed studies of sufficient subject size to enable subdivision of phenotypes are needed.

**The IL-33/IL-1RL1 pathway; potential for targeted treatment in specific asthma phenotypes?**

The genetic, functional and clinical data that we generated in our thesis and discussed above, supports that the IL-33/IL-1RL1 pathway associates with an eosinophilic, severe asthma phenotype characterized by increased expression of IL-33 and overall a decreased expression of the decoy receptor IL-1RL1a, with potential effects running via altered eosinophilic- and Th2-mediated inflammation. Also, we found initial evidence that SNPs in the *IL1RL1* route associated with specific food allergy phenotypes, although we postulate that the latter requires further replication in larger cohorts before drawing too firm conclusions.
Therefore, in light of targeted treatment strategies that we will discuss in this part I would like to focus on the specific severe asthma phenotype characterized by eosinophilic inflammation that we particularly found associated with the IL-33/IL-1RL1 pathway.
Based on our findings, we hypothesize that treatments directed at (the various members of) the IL-33/IL-1RL1 signalling route could potentially be beneficial for either prevention and/or treatment of asthma characterized by eosinophilic inflammation. However, our data also suggest that the effect of IL-33/IL-1RL1 inhibition might go beyond suppression of eosinophilic inflammation and its clinical correlates. Our conditional analyses in chapter 4 show that an association of *IL33* signals with asthma persisted after correction for eosinophil counts, which indicates that the role for the pathway extends beyond the regulation of eosinophil numbers. Thus, it will be of interest to assess asthma symptoms and lung function parameters in addition to eosinophilic inflammation in any clinical studies investigating anti-IL-33 or anti-IL-1RL1 drugs.
What could be rational approaches to inhibit the activity of this pathway? First, strategies to reduce the available IL-33 could potentially be beneficial. These could for example be in the form of monoclonal antibodies directed against IL-33 or surrogates of the IL-1RL1-a decoy receptor to capture and sequester IL-33. The latter includes a fusion protein called IL-33 Trap, formed by sST2 and the accessory protein IL-1RAcP (49). Second, inhibitors binding to the transmembrane IL-1RL1 receptor could reduce IL-33 signalling and hence diminish downstream inflammatory effects. (248,288,289). A potential point of attention for the

latter, however, could be the presence of the natural occurring soluble IL-1RL1 which might also bind to drugs directed at IL-1RL1, capturing the drug.

In the past decade, these strategies directed against the IL-33/IL-1RL1 have been tested in preclinical (animal) models of asthma and inflammatory airways disease, but also in allergic rhinitis, food allergy and eczema models. Most of these only applied one type of strategy, i.e. either anti-IL-33 or anti-IL-1RL1, but these strategies showed beneficial effect for respiratory symptoms, extent of (upper and lower) airway inflammation, severity of food allergy (prevention of anaphylaxis) and development of allergic dermatitis/eczema in these studies. (268,269,290-299). Thus, these studies show promising data that inhibition of the pathway may be beneficial in allergic disease.

Over the past few years, pharmaceutical companies have started phase I and phase II clinical trials investigating the effect of anti-IL-33/IL-1RL1 strategies in particular asthma, atopic dermatitis and food allergy (see overview table). Very interestingly, the food allergy phenotype that is studied in one of the trials is peanut allergy, and some of the clinical asthma trials include eosinophilic asthma phenotypes.

***Overview of current clinical trials directed against the IL-33 pathway in asthma and allergy-related disorders. CRSwNP= chronic rhinosinusitis with nasal polyps***

| Drug | Clinical trial number (hyperlink) | Company | Mechanism | Route of administration | Phase | Phenotype |
|---|---|---|---|---|---|---|
| **AMG-282 (RG6149)** | NCT01928368, NCT02170337 NCT02918019 | AmGen | Anti–IL-33 receptor mAb | s.c./i.v. | II for asthma, I for CRSwNP | Mild atopic asthma, CRSwNP |
| **ANB020 (Etokimab)** | NCT03469934, NCT02920021 | AnaptysBio | IL-33 antagonist/ anti–IL-33 mAb | s.c./i.v. | II | Severe asthma (eosinophilic phenotype), peanut allergy, AD |
| **GSK3772847 (CNTO7160)** | NCT03207243 NCT03393806 | GlaxoSmithKline | Anti–IL-33 receptor mAb | i.v. | II | Moderate to severe asthma |
| **SAR440340 (REGN3500)** | NCT02999711 NCT03112577 NCT03387852 | Sanofi/ Regeneron Pharmaceuticals | IL-33 antagonist/ anti–IL-33 mAb and anti- IL-4Rα | s.c. | II | Moderate to severe asthma |

First results are being reported at the moment, with signs of beneficial effects of blocking strategies of the IL-33/IL-1RL1 pathway for example for asthma control, although these reports have not been published yet by peer reviewed journals (288,289). Based on the current thesis, it would be very relevant to see whether specific eosinophilic asthma phenotypes would benefit from these blocking strategies and whether anti-IL-33 or anti-IL-1RL1 would have the preference in light of efficacy, side-effects or duration of effect, but also in several phases of airway disease (inflammation vs remodelling). In the Regeneron studies for example, the largest effect of anti-IL-33 seems to be in the subgroups of asthma patients with high blood

eosinophils (≥300 cells/microliter). Despite these first positive results, direct comparison of IL-33/IL-RL1 blocking strategies to other monoclonal antibodies directed at other cytokines, such as Dupilimab (anti-IL-13/IL-4) do not seem to show a higher efficacy of anti-IL-33/IL-1RL1 blocking strategies. More specific definition of asthma subgroups reflecting IL-33 biology, using a combination of indicators might be key in identifying anti-IL-33 responders. Based on the research presented in this thesis, I think that subgroups that would benefit from anti-IL-33 and or anti-IL-1RL1 treatment would potentially be distinguished by specific *IL33*/*IL1RL1* genotypes and soluble levels of IL-1RL1a. Potential candidate SNPs I would suggest could be the *IL33* SNP rs992969 and the *IL1RL1* SNP rs1420101, in combination with levels of IL-1RL1a in serum, with a clinical asthma phenotype characterised by eosinophilic airway inflammation, severe obstruction and early onset.

How would novel drugs targeting the IL-33/IL-1RL1 pathway be positioned in relation to other biologicals either being on the market or in development? One could write an entire review discussing the (theoretical) relevance and pros and cons of (targeting) different molecular pathways for specific asthma groups. (288,289) Such is beyond the scope of this thesis, and head-to-head direct comparative clinical studies of different biologicals would be required to guide this, which are currently lacking.

Biological therapies (monoclonal antibodies) currently on the market for treatment of (otherwise difficult to treat) asthma mainly target signalling pathways involved in type 2 inflammation, which is mediated by cytokines such as IL-4/IL-5/IL-13, and measured using biomarkers such as blood and sputum eosinophils, and fractional exhaled nitric oxide (FeNO). (300) Approved drugs include anti-immunoglobulin (Ig)E, anti-IL-5, anti-IL-5Rα and anti-IL-4Rα monoclonal antibodies (the latter blocking the IL-4 and IL-13 pathways). These have been shown to decrease exacerbation rates in study populations by ~50%, (172,301,302) but much variability in efficacy exists. Since these specific biologicals target only some of the elements of the pathways that regulate type 2 inflammation (see figure 2), not the full inflammatory patterns are addressed, potentially explaining the variability in efficacy. Alternatively, these drugs directed at specific type 2 cytokines need to be directed in very specific patient groups. For example, anti-IL-5 is suggested to be most likely relevant in late-/adult-onset eosinophilic asthma, that have high sputum or blood eosinophils (288). While therapies directed at IgE and/or IL-4/IL-13 might be more effective in patients with early-onset allergic asthma, (288,289) characterized by (high) serum levels of allergen-specific IgE and total IgE, and elevated FeNO, which is characteristic of inflammation predominantly driven by Th2 cells and/or IL-4/IL-13. (303,304)



In this light, I would hypothesize that biologicals directed at more upstream molecules, such as epithelial alarmins, including IL-33 and TSLP and perhaps IL-25, would potentially have higher efficacy in treating type 2 high asthma in a broader patient population, as multiple elements of type 2 inflammation would be targeted.

Moreover, some of these alarmins might be involved in non-type 2 driven inflammatory pathways in asthma such as Th1 and Th17 driven pathways characterized by neutrophilic or mixed granulocytic inflammation (see figure 2), and anti-alarmin therapy might find their way

in these categories of patients. (288) Of the anti-alarmins, anti-TSLP (tezepelumab) has reached the furthest stage of clinical trials at current. And indeed, its phase 2b trial suggests this anti-TSLP biological to be effective in reducing asthma exacerbation rate regardless of a patients' baseline type 2 immune status (i.e. irrespective of blood eosinophil count, level of FeNO, or serum levels of IgE, IL-5, IL-13), suggesting that it may be effective in the type 2-low asthma subtype. (305) This requires much more study however. Also whether targeted treatment directed at IL-33/IL-1RL1 would have a role in non-type 2 asthma is to be determined.

In summary, I think that treatments directed at alarmins, such as the anti-IL-33/IL-1RL1 would have a role in a broader population of type 2 high asthma patients than treatments directed at IL-4/IL-13/IL-5 or IgE, and some of the alarmins might be useful to target in non-type 2 asthma. Direct, head-to-head comparison of these novel biologicals are warranted, such as is done by Regeneron in a direct comparison of anti-IL-4/IL-13 and anti-IL-33 (NCT03387852). (288) Crucial to my opinion would be to include several specific patient groups (type 2 high/low, allergic/non-allergic) to guide a substantiated choice of targeted treatment for particular patients.

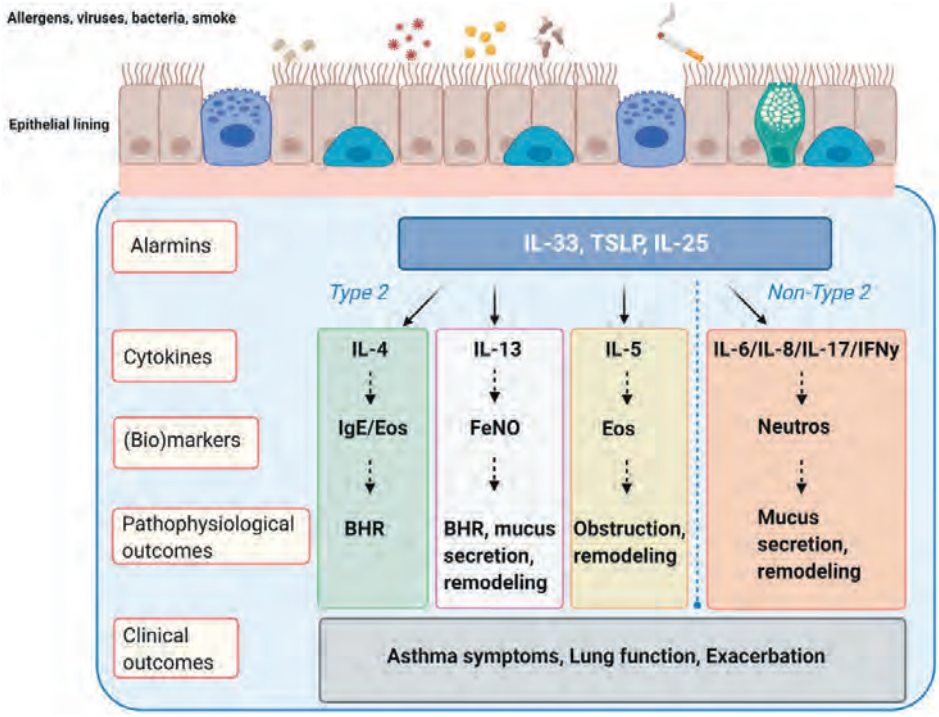


***Figure 2***

Novel biologicals might be targeted at several levels of underlying molecular pathways in asthma. Alarmins such as IL-33 and TSLP might overall be relevant targets in type 2 asthma compared to drugs directed at specific downstream cytokines. As yet alarmins have to be investigated further for their role in non-type 2 asthma. IL: interleukin; TSLP: thymic stromal lymphopoietin; IgE: immunoglobin E; Eos: eosinophils; FeNO: fractional exhaled nitric oxide; Neutros: neutrophils; BHR: bronchial hyperresponsiveness

**Important side-notes**

When moving towards interfering strategies in a molecular pathway thought to underlay certain disorders, it is very relevant to be aware of potential physiological or even beneficial/protective effects of a pathway. This would also enable us to pay attention to side-effects that could potentially arise when blocking a pathway, or at least one could attempt to predict these and include as secondary outcomes of clinical trials. However, the paper of Smith et al. (34) suggests that people expressing lower levels of IL-33 due to a rare genetic *IL33* variant are protected from asthma, but apparently without any other comorbidity, at least within the investigated scope. Whether or not that could be truly predictive of (lack of) side-effects from anti-IL-33 treatment is to be found out in clinical trials. Very relevant, therefore, could be to predict potential side-effects from genetic studies, as *IL33* SNPs have also been associated with auto-immune diseases, cardiac disorders and neurological diseases.

In that line, functionally it is becoming more and more evident that IL-33 could have protective effects in the cardiovascular system, including prevention of cardiac fibrosis in cardiac pressure overload hypertrophic models or in prevention of atherosclerosis (306-309) However, in more acute inflammatory cardiac disorders, such as myocarditis and eosinophilic pericarditis IL-33 showed pathogenic effects (310). Also in the central nervous system IL-33 has a dual role; it may be protective in ischemic stroke, spinal injury and Alzheimer dementia models, but has pathogenic effects in infectious meningitis and in auto-immune encephalomyelitis. (311-321)

Likewise, for the gastro-intestinal tract the IL-33/IL-1RL1 axis seems important for the maintenance of the epithelial integrity of the gastrointestinal tract, but its effect dependent of the phase of disease, for example in colitis. Presence of IL-33 at early stage of colitis increased colon inflammation, while treatment with IL-33 in the recovery phase reduced colitis. (322-325) A time/disease phase-dependent effect of activation of the IL-33/IL-1RL1 pathway might be in analogy to what we found for soluble IL-1RL1a in the pulmonary system; whilst high levels of IL-1RL1a seem to be associated with protection of development and presence of asthma, during asthma exacerbation higher levels of IL-1RL1a are associated with severity of exacerbation.

The above underlines that no treatment strategy is straightforward; effects may depend on type of disease, phase of disease and severity of disease, and so will be its potential side-effects. Genetics could be predictive of (side)effects, but true evidence would arise from more mechanistic studies, hand-in hand with careful design of future (phase III and IV) clinical trials that should be aware of and include the measurement of potential side-effects based on current and ongoing knowledge of the (patho)physiological role of the IL-33/IL-1RL1 pathway.

## Future Perspectives

Future studies on several levels of evidence would be relevant to better understand the role of this pathway for disease development, disease control and how interfering strategies should be directed to limit inception, improve control and potentially even lead to remission of disease. Overall, I think that the future should hold a combination of (functional) genetic and bioinformatics studies to unravel causative functional variants at the *IL33* and *IL1RL1* loci, in combination with mechanistic studies using clinical samples from selected patient groups. This could guide the treatment of drugs interfering in the IL-33/IL-1RL1 pathway and enable the selection of patients that would benefit most from such strategies based on a combination of genetic risk factors (e.g. *IL33* and *IL1RL1* SNPs, molecular/cellular characteristics (e.g. eosinophilic, type 2 high) and clinical features (i.e. severe asthma with low lung function.)

*On a functional genetic level*, one major advance would be to design studies to find out the SNPs that are causally involved in dysregulating the balance of IL-33/IL-1RL1. Predictive algorithms of non-coding and coding effects of asthma/allergy-associated SNPs at the *IL33* and *IL1RL1* loci could guide the prioritization of SNPs for such functional studies. Alternatively, we may move to the investigations of populations in which different LD patterns exist at the *IL33* and *IL1RL1* loci to help dissect distinct signals, as was recently shown in studies of African-America children. (250) Functionally, for *IL33* and *IL1RL1* strategies to find genetic variants causally involved in expression regulation, like 3C or 4C strategies, and/or reporter gene constructs would be useful in this. For *IL1RL1* also single-nucleotide mutation strategies in coding areas, respecting naturally occurring haplotypes for SNP-SNP interactions would be useful strategies to further unravel the role of coding SNPs for IL-1RL1 mediated inflammatory pathways. This should go hand-in-hand with single cell eQTL/ATAC-Seq studies, to unravel which genetic signal is active in what cell type. In this light, whole genome sequencing techniques are useful, to also look at rare and structural variants that would have functional consequences, as well as to look at whole-genome epigenetic patterns linked to expression of *IL33* and *IL1RL1*. This would teach us what (type of) genetic signals are crucial for the activity, expression and (patho)physiological function of the IL-1RL1 pathway, and how disruption of it would lead to disease. This could then be applied in the clinical context, for example by using specific functional genetic variants as predictors of disease and treatment response, and to guide direction of treatments at specific parts of the IL-1RL1 signalling pathway in specific cell types.

*Mechanistically*, very interesting would be to study epithelial-mediated effects on inflammation by using approaches of co-culture models (for instance coculture of epithelial cells and inflammatory cells) especially using 3D culture models characterized by epithelial differentiation such as the air-liquid interface (ALI) and epithelial organoid models. This could also help to understand whether epithelium is (only) a producer of IL-33 or also a responder to IL-33 in interaction with other (e.g. inflammatory) cell types. In addition, studying effects on remodelling using more structural cells, by investigating effects of IL-

33 and IL-1RL1 on fibroblasts, smooth muscle cells and in more versatile 3D models such as lung-on-a-chip models and culturing cells in 3D printed extracellular matrix would be crucial to understand the IL-33/IL-1RL1 effects beyond inflammation.

Furthermore, I think that bioinformatic strategies in combination with sophisticated single-cell techniques could be informative for unravelling key cell types and states involved in IL-33/IL-1RL1 signalling, to link this to the pathogenesis of altered airway function, intestine function or skin function in respectively asthma/rhinitis, food allergy and eczema. Indeed, by extending the latter: key would be to study this pathway in several organ systems, as one could then get a better idea of potential (side-) effects when developing interfering strategies of the IL-33/IL-1RL1 route.

Importantly, being also one of the main messages that has come up in this thesis; crucial in *clinical studies* would be to carefully define the (sub)phenotype that is studied, based on clinical parameters, genetic markers and expression levels, to be able to find key roles of the IL-33/IL-1RL1 pathway in asthma and allergy pathogenesis of specific endotypes.

Lastly, although the role of several forms of IL-1RL1 RNA and protein are being recognized at current, an evolving and very relevant future area of research would be to study several isoforms of IL-33, as well as to try to unravel their roles in the nuclear, cytoplasmic, and extracellular compartments, to enable further balancing of interfering strategies of IL-33 targeting. Likewise, several (iso)forms of both IL-1RL1 and IL-33 might hold different clinical potential for predicting different forms of disease.

So, overall, there is no single road to find the best prescription for a patient, this would rather benefit from multiple roads: further unravelling disease mechanisms using basal scientific studies as well as from clear clinical study of subphenotypes of disease, such that both molecular and clinical characteristics could be combined in specific endotypes that could be selected for tailored targeted treatment strategies.