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Potential biomarkers for the prediction of childhood wheeze: Insights into new gene regulation mechanisms of the innate immune system at birth

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INHALTSVERZEICHNIS

1. INTRODUCTION

Allergic diseases are the most frequent chronic diseases in childhood [1] and one important manifestation is asthma. This pulmonary disease presents with airway hyperresponsiveness leading to airway obstruction and shortness of breath.
Patients suffer from acute respiratory distress with expiratory wheeze that can lead to extreme anxiety, tachycardia and - if untreated - can result in the life-threatening status asthmaticus [2].
Although its pathogenesis is not completely understood, many factors contributing to the development of asthma have been found including environmental exposure [3], nutrition [4], genetic and epigenetic mechanisms [5].
World-wide, an estimate of 334 million people are affected by this disease with the prevalence in children having increased over the past decades up to 14 percent [6]. As asthma comes along with the potential threat of exacerbations and a life-long need for treatment, especially an early onset of the disease – childhood asthma – puts a burden on both the little patients with their families and the public health care system.
Many approaches have been established in order to control asthma symptoms and to prevent exacerbations. However, over the past decades it became clear that asthma is rather a clinical syndrome than a 'simple' disease, paying tribute to its many different manifestation forms.
In early life, asthma cannot be diagnosed due to the limited compliance of young children. Therefore, the term `wheeze´ was established for young patients with asthma-like symptoms. Childhood wheeze is known to be a crucial risk factor for asthma development [7]. However, research is recently focusing on the heterogeneity of childhood wheeze and the different outcomes later in life. While some children develop asthma, others are likely to outgrow their symptoms [8].
In this context, the need for a more individual therapy has increased, leading to the necessity of a more precise classification of affected patients. Therefore, defining different phenotypes and finding potential biomarkers has become of recent interest.

1.1. Asthma classification

1.1.1. Phenotyping wheezing infants

Many approaches for classifying childhood asthma have been established. To date, one common classification is dividing children into either allergic or non-allergic asthma type. Whereas allergic asthma is characterized mainly by specific sensitization and high IgE-levels, non-allergic asthma features neutrophilic inflammation [9].
Due to the limited compliance in lung function testing especially of younger children, the definitive diagnosis 'asthma' cannot be made before the age of 5 years, leaving a classification gap for younger children with asthma-like symptoms. Therefore, so-called wheeze phenotypes have been described for preschool-age children. These infant wheeze phenotypes are defined by two approaches: by a clinical and an epidemiological perspective.

Clinical phenotyping is based upon criteria such as symptom triggers, their association with infections, patient history and allergic sensitization as well as with frequency and severity of symptoms. Considering all those clinical features, the resulting phenotypes mainly focus on *what* the symptoms are triggered by, *how* patients respond to treatment and *what other allergic* symptoms they have [10]. These phenotypes include, for example, multitrigger wheezing, unremitting, frequent or episodic wheezing.
An additional clinical approach is driven by the predominant cell type found in patient samples, like peripheral blood samples or bronchoalveolar lavage fluid resulting in an eosinophilic phenotype (with mainly T_H2 cells and eosinophilic granulocytes) and a neutrophilic phenotype (with mainly T_H1/T_H17 cells and neutrophilic granulocytes).
In contrast, epidemiological considerations focus on *variation with time* and are driven by data-based latent class analysis. This analysis is part of the structural equation modeling that identifies subgroups of cases in multivariant categorical data. In asthma research, this leads to phenotype definition such as early transient, persistent and late-onset wheezing [11].
Those two approaches have been compared lately, showing that they are highly correlated and that clinical phenotyping is well supported by epidemiological phenotyping [12].
When trying to define different phenotypes, differentiation between endotypes has become more and more important.

1.1.2. Endotyping childhood wheeze

The term endotyping describes the approach to define different subgroups of a disease, in this case wheezing infants, according to molecular mechanisms contributing to its pathogenesis. Gaining a better understanding of the molecular mechanisms leading to childhood wheeze - and the potential development of asthma later in life - might be a way to provide a more personalized and therefore more effective treatment strategy. This could help especially those patients who do not respond well to today's treatment guidelines. With our current knowledge we are not satisfactorily able to explain those treatment failures and the reasons why they fail, suggesting that the complexity of asthma and the resulting patients' heterogeneity need further investigation from a new perspective.
To classify endotypes, information from molecular pathways is put together: genome-wide association studies tried to find single nucleotide polymorphisms correlated with asthma risk and protection [13], gene expression on RNA level has been investigated along with its regulation by micro RNA [14] and protein levels have been studied by looking at differences in cytokine levels in asthmatic patients and healthy controls. All these efforts result in new insights into the disease's pathology and may help to find new promising therapeutic targets.
In this project, focus has been put on identifying potential new genes of the innate immune system associated with an increased risk for asthma at the earliest possible time point, directly after birth. The analysis at said time point might contribute to finding potential new biomarkers for the prediction of subsequent asthma development.

1.2. Immune system

1.2.1. Innate immune system

Every day, our immune system has to face an enormous number of antigens resulting in the need of providing a very quick 'first-line' response to potential harming microorganisms.
In order to ensure a rapid response, the human body has brought up a wide range of cells that quickly but unspecifically fight potential threats like pathogens, necrotic and apoptotic cells or tumor cells: the innate immune system.
This initial immune response is highly conserved over evolution and is provided by monocytes/macrophages, dendritic cells, natural killer cells, eosinophil, basophil and neutrophil granulocytes and mast cells, with each cell type having a specific function within the immune system (see Figure 1).

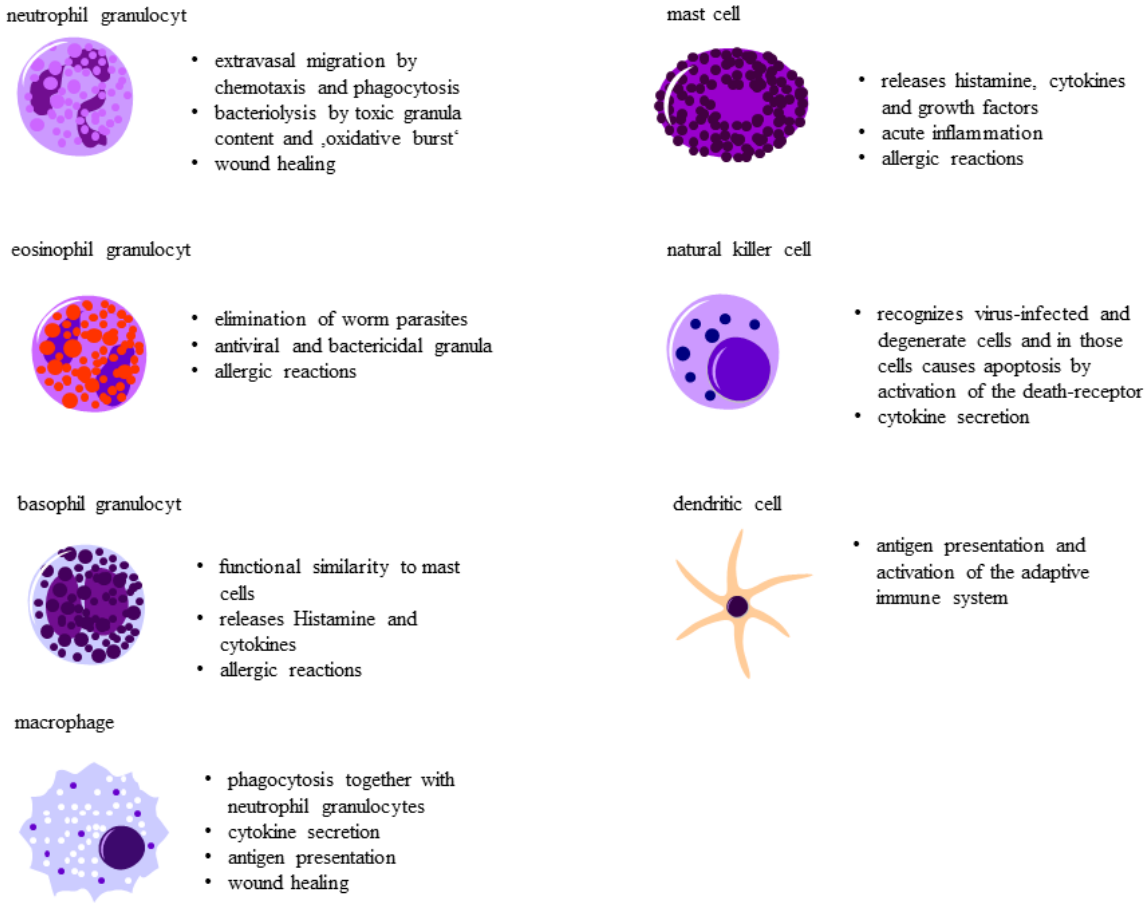


Figure 1: Cells of the innate immune system.
Phagocytosis= engulfing of a solid particle into an intracellular vesicle called phagosome followed by an enzymal digestion
Chemotaxis= cell movement in response to chemical stimuli like cytokines
Cell functions taken from Lüllmann-Rauch [15]

In order to fulfill their function, it is essential that the cells of the innate immune system are able to distinguish between 'foreign' cells and natural body components. For that determination so-called pathogen-associated molecular patterns (PAMPs) play a crucial

role. PAMPs are highly pathogen-specific target for innate immune cells as they are only produced by microorganisms, invariant between those of a given class and essential for microbial survival [16].
The recognition of PAMPs is provided by the pattern recognition receptors (PRRs) that play a major role in shaping the innate immune response.
Additionally, the innate immune system senses cell damage like necrosis with the help of damage-associated molecular patterns (DAMPs). Those molecular patterns are mainly formed by intracellular molecules like RNA or S100 proteins with their extracellular appearance signalling cell lysis.
When identifying PAMPs or DAMPs, cells of the innate immune system, especially macrophages and neutrophilic granulocytes, rapidly trigger a pro-inflammatory immune response leading to the liberation of various cytokines like Interleukin-1, Interleukin 8, tumor-necrosis factor α (TNFα) and Interferon γ (IFNγ).
Another important function of the innate immune system beside the 'first-line' response is the presentation of antigens on major histocompability complexes (MHCs). Especially the phagocytes, including dendritic cells, macrophages and neutrophil granulocytes, fulfill this function leading to the recognition of the invaded pathogen by the adaptive immune system.
Although the innate immune system is essential for the human immunity, an aberrant activation and dysregulation can result in inflammatory and atopic diseases [17-19].
It has been shown that some cell types of the innate immune system, especially neutrophils and eosinophils are enriched in children with asthma[20] highlighting the potential role of those cell types in asthma manifestation.
Furthermore, Boeck et al. found differently expressed innate immune pathways in different childhood asthma phenotypes [21].
Asthma might also be combined with the reduced ability to fight a systemic virus infection caused by an inadequate response by the innate immune system [22].
While the important impact of the adaptive immune system on asthma has already been shown, the question arises by what mechanisms this adaptive immune response is modulated [23-25]. Therefore, recent research has put focus on the impact of the innate immune system [26].
Dendritic cells play a major role in antigen presentation and triggering of the adaptive immune response by priming of naïve T cells. This priming step is crucial for the development of either a T_H1 or T_H2 cell response resulting in different cytokine milieus that have an impact on the development of asthma. Only mature dendritic cells are able to stimulate naïve T cells. As the shift of naïve T cells towards either T_H1 or T_H2 cell response is essential for asthma development and mediated by mature dendritic cells, the maturation process of the dendritic cells has to be tightly regulated. Dendritic cell maturation is shaped by the innate immune system resulting in a crucial role of the innate immune system in asthma pathology [24].
In order to further understand this expected role, genes associated with toll-like receptors, RIG-I like receptors, C-type lectin receptors, the immunoproteasome and the inflammasome – that all shape the innate immune response – were analyzed in this project.

1.2.2. Adaptive immune system

Following the rapid initial immune response provided by the innate immune system, the adaptive immune system raises a more target-orientated and therefore specific immune response. However, this response takes 4-7 days to be established [27].
Additionally, the adaptive immune response has a memory function.
Upon activation and priming, two cell types mediate adaptive immunity: B cells and T cells, with their different subtypes that can be differentiated by their specific cell surface molecules, the so-called cluster of differentiation (CD) antigens and their function (see Figure 2).
Naïve B cells mature into plasma cells, that repel pathogens by releasing specific immunoglobulins (Ig), the so-called antibodies and into said memory cells which provide a quicker response in case of a new infestation by the same pathogen.
T cells form the T effector cells that recognize and kill infected cells and T helper cells and regulatory T cells that shape and modulate the immune response.

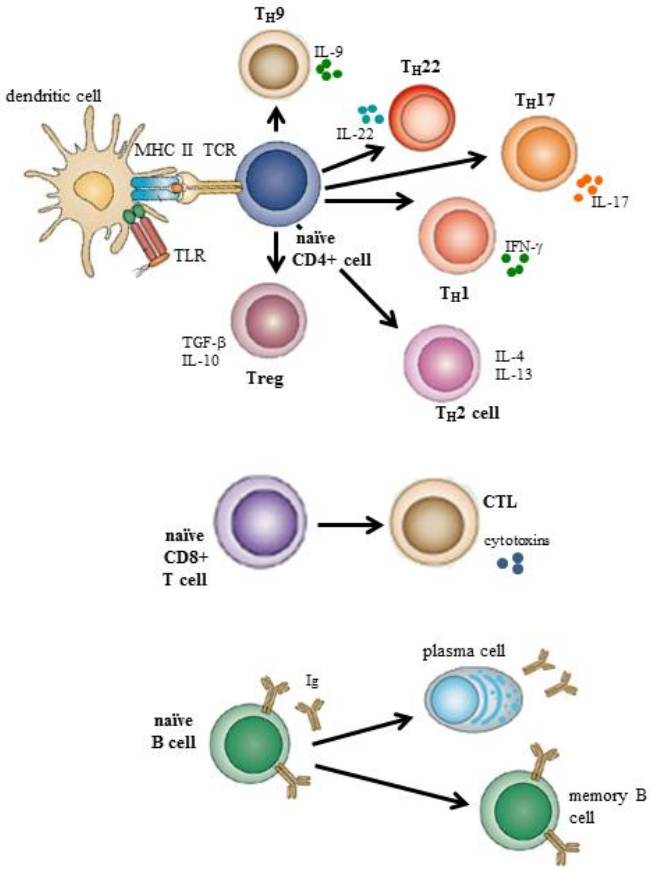


Figure 2: T and B cell differentiation.
Modulated by dendritic cells, naïve CD4+ Tcells (Cluster of Differentiation 4) differentiate to various subtypes of T_{Helper} (T_H) cells that secrete cell specific Interleukins (IL), Interferon-γ (INFγ). Additionally they can turn into regulatory T cells (T_{reg}) that produce among others transforming growth factor β (TGFβ). Naïve CD8+ T cells mature into cytotoxic T lymphocytes (CTL) producing various cytotoxins in order to kill infected cells. Naïve B cells can turn either into immunologically active plasma cells producing immunoglobulins (Ig) or to a small amount into memory B cells providing a quicker response to reinfection by a known pathogen. Figure adapted from Klucker, Raedler [28].

Various studies have already linked the specific subtypes of adaptive immune cells to different asthma manifestations, highlighting the important role of T_H1/T_H17 [29], T_H2 [18] and T_{reg} [9, 30, 31]in the pathogenesis of childhood asthma.
Over the past decades, asthma has been seen as mainly a T_H2 disorder [25] with the imbalance between T_H2 and T_H1 cells contributing to the asthma development. Recently, there has been growing evidence that not only T_H2 cells but also other T cell subtypes like T_H17 cells and T_{regs} have a crucial impact on the disease's pathology. For example, it has been shown that the acetylation of T_{reg} genes differ between children with an asthma risk and healthy controls [32].

1.3.NFkB signalling and gene regulation

The nuclear factor 'kappa-light-chain-enhancer' of activated B-cells -signalling pathway (NFκB signalling pathway), expressed in almost all mammalian cell types [33], is known to play a crucial role in the development of inflammation, modulation of the innate immune response and in the pathology of asthma [34].
The protein complex consists of NFκB1 (p105/p50), NFκB2 (p100/p52), RelA (p65), RelB and c-Rel [35]. They all have an N-terminal Rel homology domain (RHD) that enables them to sequence-specifically bind DNA.
Upon activation, the subunits of the NFκB complex form diverse homo- and hetero-dimers that transfer to the nucleus and lead to the transcription of pro-inflammatory signalling pathway genes.
In the unstimulated cell, the NFκB complex is inhibited by its antagonist, the IκBs (Inhibition of kappa B) that bind to the RHD and retain the NFκB dimers in the cytoplasm. Cell stimulation leads to the activation of the IκBα kinase complex (IKK) that liberates the NFκB subunits by phosphorylation and degradation of the IκBs.
Three proteins belong to the IKKs: IKKα, IKKβ and IKKγ/nemo.
There are two different pathways of NFκB stimulation: the classical or canonical pathway and the alternative pathway (see Figure 3).
Many pro-inflammatory cytokines and PAMPs activate the classical pathway by binding to tumor necrosis factor (TNF) receptors and Toll-like receptors (TLR). The classical pathway is then mainly mediated by the IKKβ/γ leading to the liberation and dimerization of RelA:p50 and c-Rel:p50 dimers and plays a crucial role in the innate immune system [36].
In the alternative pathway, a small subset of TNF family members activate IKKα via the NFκB inducing kinase [37]. The alternative pathway seems to play a role in modulating the adaptive immune response as it has an impact on the spleen development and organization [36].

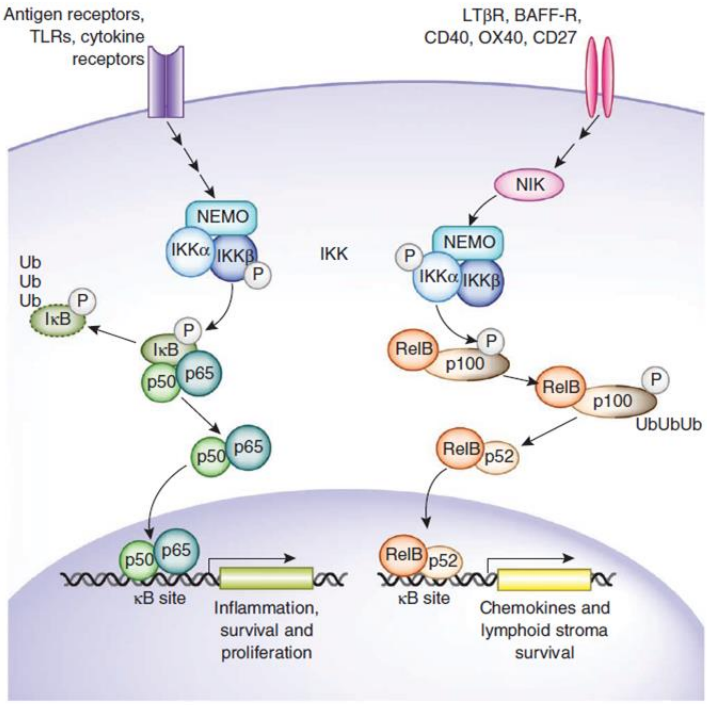


Figure 3: Canonical (left) and alternative (right) pathway of NFκB activation. In the canonical pathway, through activation of various receptors, the IKK (IκBα kinase complex) is activated and phosphorylates IκB (inhibitor of NFκB). This leads to the ubiquitination and degradation of IκB resulting in the liberation and dimerization of the NFκB subunits which then transfer to the nucleus and induce gene expression. The alternative pathway is mediated through the activation of NIK (NFκB inducing kinase), also resulting in the dimerization of NFκB subunits and is induced by a small number of TNF family member. Figure by Gerondakis, Fulford [38]

By now, not all details of the NFκB signalling pathway are fully understood. However, various studies highlight its important role in the regulation and modulation of the immune system. Additionally, research has focused on the NFκB dysregulation in autoimmunity and inflammatory diseases such as asthma [39] leading to further understanding of the known therapeutic effect of NFκB inhibition [40].
Furthermore, it has been shown that a protective effect on asthma development is mediated through the limitation of NFκB pathway activity by A20 [41, 42]. These findings indicate that the dysregulation of NFκB associated pathways contribute to the pathology of asthma.
Therefore, it seems highly interesting to further investigate what causes the dysregulation that may contribute to the development of asthma. In this project, genes that both influence the NFκB signalling pathway and are partly already linked to asthma development were analyzed in cord blood cells to investigate whether their expression differs from healthy children already in the very beginning of life.

1.4. Genes of interest

1.4.1. Toll-like receptors

The toll-like receptor (TLR) family is a large group of the so-called pattern recognition receptors (PRR) and consists of 10 known TLRs in humans (TLR1-10) expressed mainly on antigen presenting cells.

TLRs play a crucial role in the recognition of PAMPs, the initiation of the innate immune response and the orchestration of the following adaptive immune response [16, 43]. Upon stimulation, TLRs use a wide range of signalling pathways to activate cells of both the innate and adaptive immune system (see Figure 4). Those pathways can be divided into receptor-specific and shared pathways. One pathway that seems to be shared by all TLRs is the activation of NFκB as demonstrated in Figure 4.

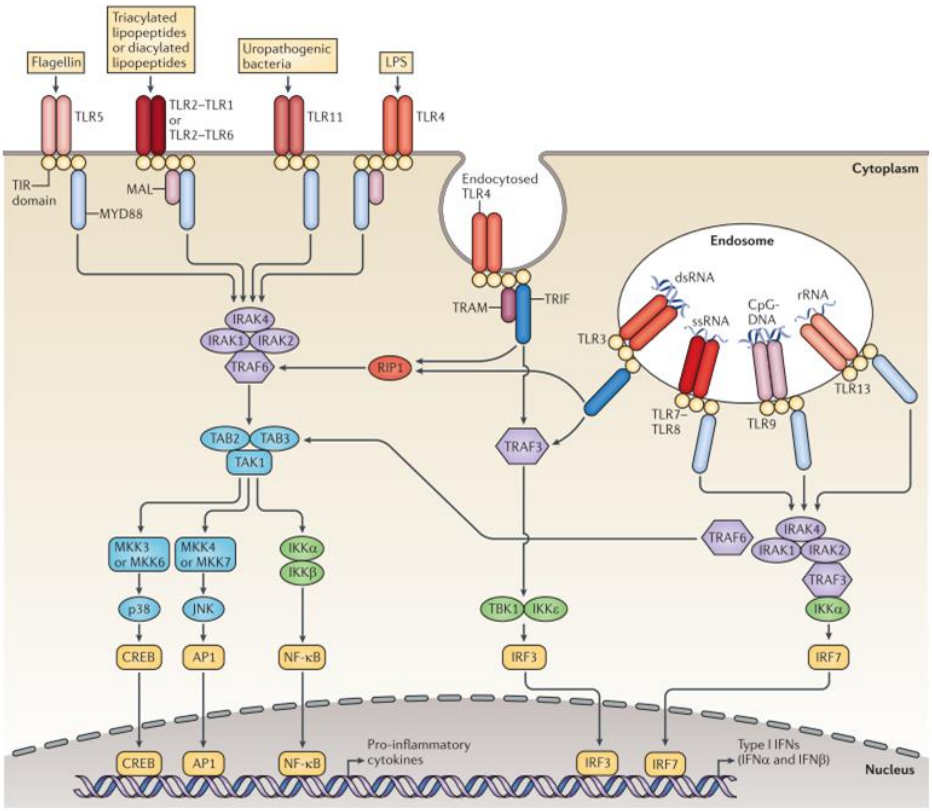


Figure 4: TLR signalling pathway. Upon activation, TLRs trigger an immediate immune response by, among other things, the activation of the NFκB signalling pathway via IRAK and TRAF6. Figure by O'Neill, Golenbock [44]

Some TLRs, like TLR4 and TLR2 have already been linked to the development of atopic diseases [45-47]. However, the role of other TLRs like TLR5 and TLR7 in the disease pathogenesis is not fully understood and of growing interest [48]. Table 1 shows the analyzed TLRs in this project.

Table 1: Genes of interest of the Toll-like receptor family

Gene	*Name*	*Location and function*	*Relevance for this project*
TLR5	Toll-like receptor 5	Cell membrane receptor for the recognition of lipid structures and flagellin	• TLR5 expression has shown to be downregulated in lymphocytes of asthmatic patients [49] • Flagellin has been shown to play a role in the sensitization to indoor allergens priming allergic asthma [50]

TLR7	Toll-like receptor 7	Intracellular receptor of the endosomal membrane Recognizes the nucleic acids of both virus and bacteria, specifically the (ss)RNA	• TLR7/8 are potential risk genes for the development of asthma and other atopic diseases [51] • Adolescents with asthma show a reduced TLR 7 function [22] • Stronger TLR7/8 response was identified in PBMCs of children with non-infectious asthma exacerbation [47]

1.4.2. RIG-I like receptors

Another group of PRRs of growing interest are the so-called retinoic acid-inducible gene I (RIG-I) –like receptors (RLRs). RLRs have just recently become the focus of investigation and therefore their role in the immune system remains yet to be fully understood.
This family consists of three receptors: RIG-I, melanoma differentiation associated gene-5 (MDA-5) and laboratory of genetics and physiology 2 (LGP2) that all sense viral double-stranded RNA [52].
They all contain a DExD/H RNA helicase domain along with two caspase activation and recruitment (CARD) domains (LGP2 has only one card domain) and are located in the cytoplasm. Additionally, RIG-I contains a repressor domain. RLR signalling leads to the activation of MAP kinase, IRF and NFκB pathway [53]. Therefore, the following genes of this pathway were investigated.

Table 2: Genes of interest of the RIG-I like receptor family

Gene	*Name*	*Location and function*	*Relevance for this project*
RIG-I (DDX58)	Retinoic acid-inducible gene I	Intracellular receptor for the recognition of viral RNA, specifically (ds)RNA	• Key regulator of innate immune response [52]
MDA-5 (IFIH1)	Melanoma differentiation associated gene-5	Intracellular receptor for the recognition of viral RNA, specifically (ds)RNA	• Key regulator of innate immune response [52] • Plays a role in the initiation of airway inflammation after rhinovirus infection in mice [54]

1.4.3. C-type lectin receptors

A third group of PRRs is formed by the C-type lectin receptors (CLRs) which recognize carbohydrate ligands. CLRs are expressed on almost all cell types and can be divided into 17 groups based on their different characteristics [55].Shared pattern of this group are the calcium dependent function, a stalk region, a transmembrane region, a signal transduction region and an extracellular carbohydrate recognition domain (CRD). Based on their CRD they can be divided into two groups: the Dectin-1 cluster with just an extracellular CRD and the Dectin-2 cluster with an additional cytoplasmic CRD [55].

Upon stimulation, the CLRs trigger an activation of the NFκB signalling pathway (see Figure 5). Therefore, CLRs have an impact on shaping the innate immune response [55], the initiation of airway inflammation [56] and might play a role in the development of allergic diseases.

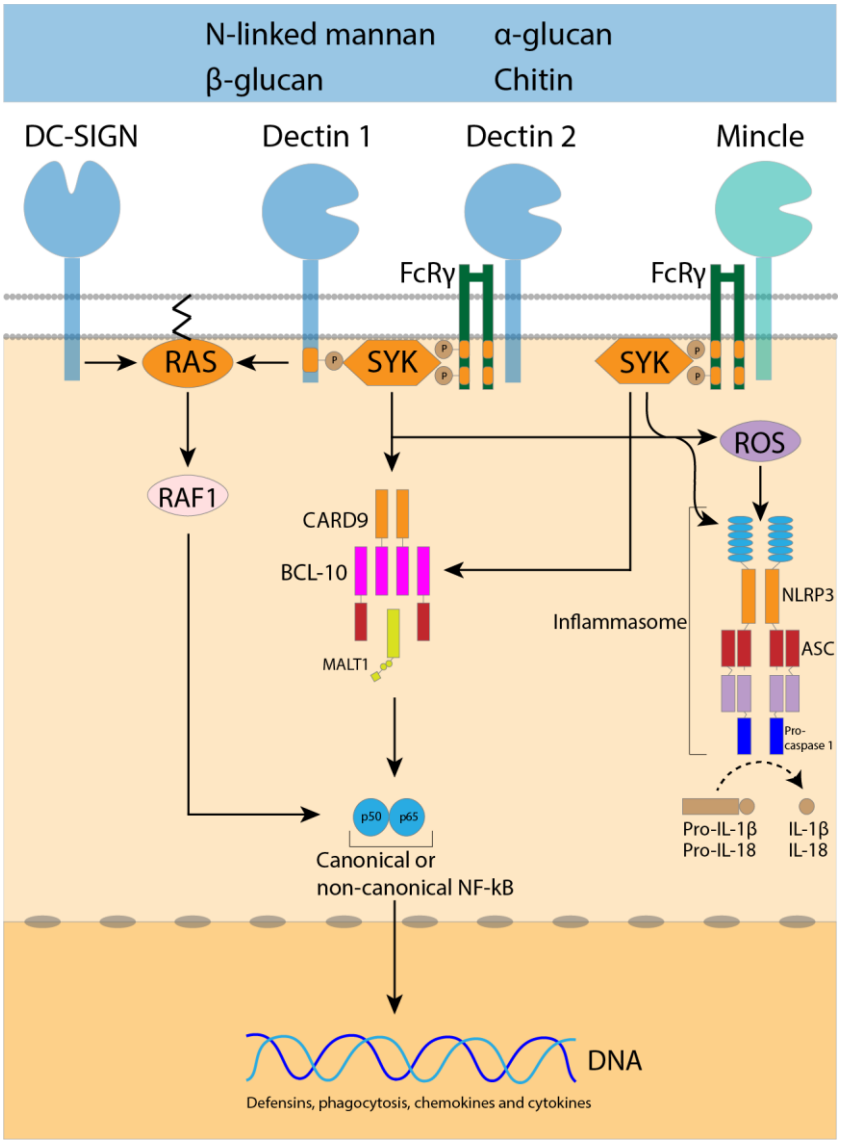


Figure 5: CLR signalling pathway. Stimulation of the CLRs leads to either a direct signal (DC-SIGN, Dectin1) or an indirect signal vial FcRγ chain (Dectin2, Mincle) resulting in the activation of NFκB and NLRP3 inflammasome. Figure adapted from Romani [57]

Table 3: Genes of interest of the CLR family

Gene	*Name*	*Location and function*	*Relevance for this project*
Mincle (CLEC4E)	C-type lectin domain family 4 member E	Cell membrane receptor that recognizes DAMPs and cord factor, a component of the cell wall of *Mycobacterium tuberculosis*	• Mincle induces IL-1 and IL-6 leading to the development of a T_H1 and T_H17 phenotype in human and mice [58]
Dectin1 (CLEC7A)	C-type lectin domain family 7 member A	Cell membrane receptor for the recognition of glucans of fungi, bacteria and plants	• Modulates NFκB signalling pathway [55] • Has an impact on the development of non-atopic asthma associated with damp buildings [59] • Plays a role in house dust mite induced allergic airway inflammation in mice [60]
Dectin2 (CLEC6A)	C-type lectin domain family 6 member A	Cell membrane receptor for numerous endogenous and exogenous ligands	• Plays a role in sensing of house dust mite and the following aberrant airway inflammation [56] • CLRs might play a role in the development of allergic asthma [61]

1.4.4. Immunoproteasome

In unstimulated cells, the standard proteasome consisting of the constitutively active β_1, β_2 and β_5 subunits is expressed and forms a cytosolic protein complex that cleaves ubiquitinated proteins into small fragments which are then presented by the major histocompatibility complex I (MHC I). The presentation on MHC I enables the activation of innate immune cells, especially the natural killer cells.
Upon activation by inflammatory signals, there's a shift in the gene expression leading to the production of different proteasomal subunits, the so-called *i*-units (β_1i, β_2i and β_5i). These subunits form a special type of proteasome, the immunoproteasome.
Immunoproteasomes are more active than the standard proteasomes and provide slightly different peptide fragments [62]. Additionally, they shape the T cell immune response as they have been reported to play a role in the T cell expansion [63]. The impact of the immunoproteasome on NFκB signalling is discussed controversially with recent evidence for its important modulating role [64].

Table 4: Genes of interest of the immunoproteasome

Gene	*Name*	*Location and function*	*Relevance for this project*
LMP2	Low molecular mass polypeptide 2	Forms the β_1i subunit of the cytosolic immunoproteasome	• The immunoproteasome might have a major impact on the NFκB signalling pathway [64]

LMP7	Low molecular mass polypeptide 7	Forms the β_5i subunit of the cytosolic immunoproteasome	• Patients with a LMP7-mutation have lower immunoproteasome content and show a spectrum of auto-inflammatory diseases that implicate aberrant NFκB signalling [64] • LMP7 deficiency and inhibition suppresses Th_1 and Th_{17} but enhances T_{reg} differentiation [65]

1.4.5. Inflammasome and Interleukin-1 receptor I

The inflammasome is a group of cytosolic protein complexes composed of nod-like receptor (NLR) proteins –a PRR subfamily–, an apoptosis-associated speck-like protein containing a CARD (ASC) domain and caspase-1. These components form different subfamilies, with different molecular structures, of inflammasomes like the AIM2, the NLRP1, the NLRC4 and the NLRP3 inflammasome. The inflammasome senses a wide range of stimuli, like PAMPs and DAMPs and by modulating the caspase-1 activity coordinates the subsequent cell response [66]. Caspase-1 cleaves the inactive Interleukin-1β (IL-1β) and IL-18 pro-forms into active cytokines that are known to play a crucial role in triggering pro-inflammatory signalling pathways [67, 68] and the regulation of T_H cells [69]. Both cytokines are also induced by the NFκB pathway, providing a link between inflammasomal and NFκB signalling.

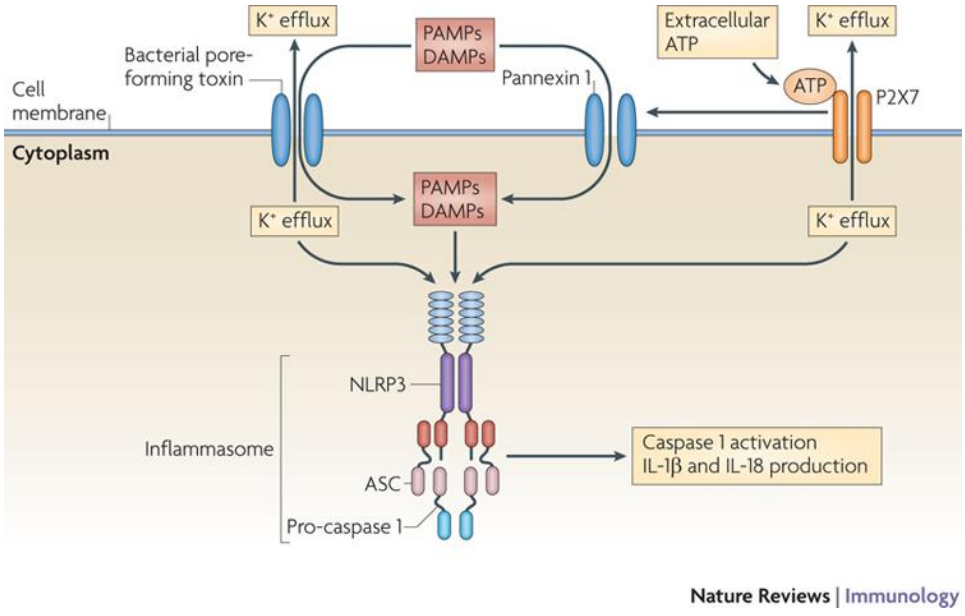


Figure 6: NLRP3 signalling pathway. Upon activation by PAMPs and DAMPS the NLRP3 inflammasome oligomerizes leading to the auto-activation of caspase-1. Figure by Tschopp and Schroder [70].

Among this family, the NLRP3 inflammasome has become of special interest as there is growing evidence for its role in airway inflammation [68, 71].

The cell-membrane receptor Interleukin-1 receptor type I (IL-1R1) is activated by IL-1α and IL-1β and further mediates the signal. In contrast, IL-1R2 attenuates the IL-1α and IL-1β signal.
Therefore, the expression of following genes was determined.

Table 5: Genes of interest of the NLRP3 inflammasome and IL-1R1 axis

Gene	*Name*	*Location and function*	*Relevance for this project*
NLRP3	NOD-like receptor family, pyrin domain containing 3	Protein of the cytosolic NLRP3 inflammasome, that upon PAMP and DAMP sensing modulates the innate immune response by activating caspase-1	• Modulates airway inflammation [68] • elevated in patients with neutrophilic asthma [71]
CASP1	Caspase-1	Intracellular enzyme that cleaves the immature pre-IL-1β and pre-IL18 into biologically active cytokines	• IL-1β and IL-18 play a crucial role in asthma development [67, 68]
IL-1R1	Interleukin-1 receptor type I	Cell membrane receptor for IL-1α and IL-1β	• Contributes to the development of HDM-related asthma in murine model [72] • Has been associated with severe asthma in humans [73]

1.4.6. Overview of the analyzed signalling pathways

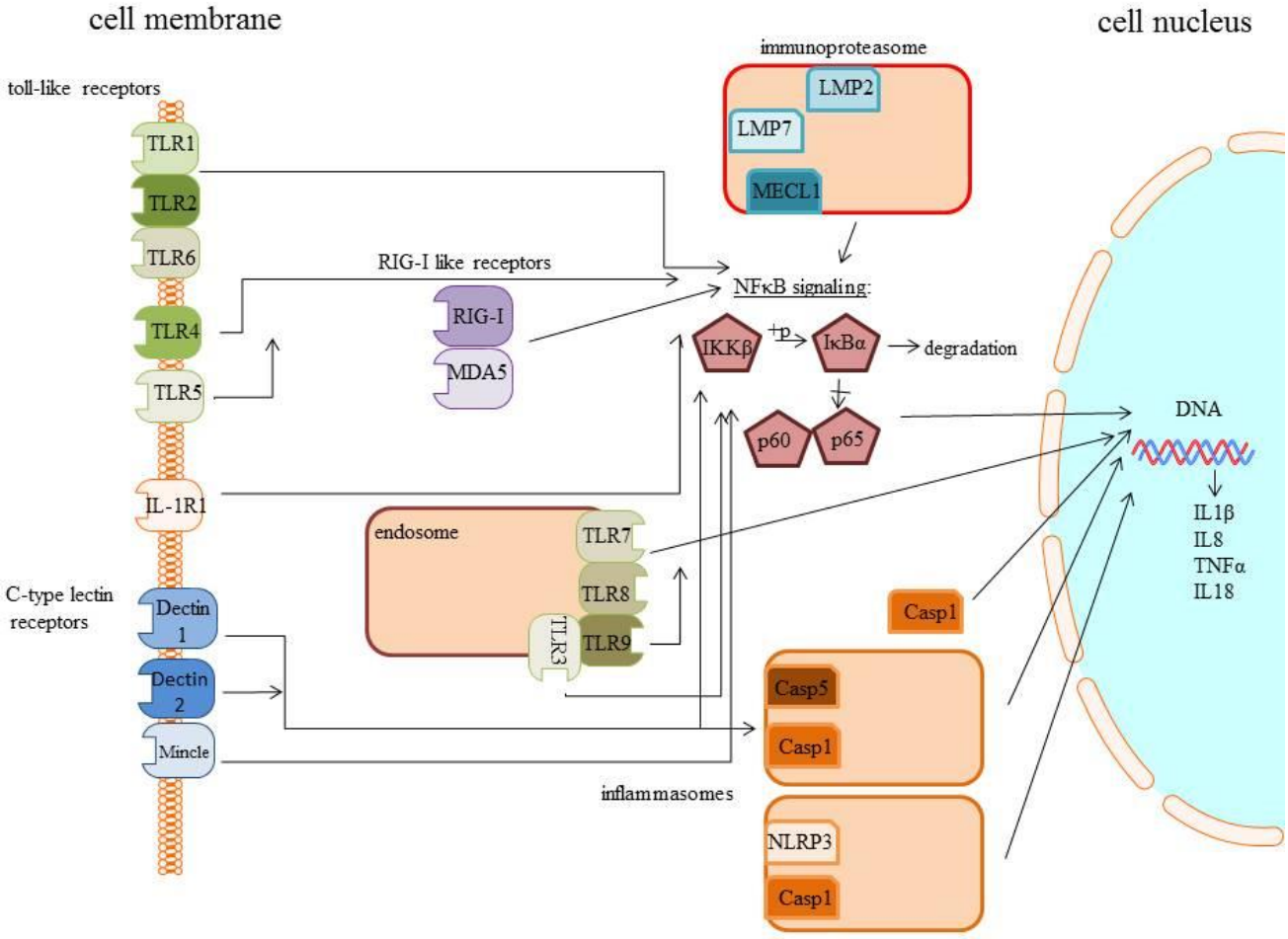


Figure 7: scheme of analyzed signalling pathways, simplified
Figure designed with motifolio.com

2. AIM OF THIS PROJECT

Over the past decades, it became clear that asthma is a much more heterogeneous disease than assumed leading to the need of a more specific classification of patients.
Even though some factors contributing to the early onset of this disease like environmental factors, nutrition and smoke exposure have been discovered, many questions about the genetic and epigenetic influence remain to be clarified.
Many approaches have been established in order to provide said classification.
In this context analysis of variation in the expression of defined genes – acting as potential new biomarkers – can contribute to a more detailed classification.
One promising approach when trying to define potential candidate genes is looking into the disease's molecular pathomechanism as affected children are likely to express genes, especially ones related to asthma development, differently from healthy children. One important pathomechanism is the chronic airway inflammation signs of which can also be detected in peripheral blood.
Inflammatory processes play a key role in the development of asthma as their dysregulation leads to the imbalance of cytokines contributing to the pathogenesis of asthma.
The innate immune system plays a crucial role in asthma development by triggering those inflammatory signals and shaping the adaptive immune response. Among others, the inflammatory signals are mediated by Toll-like receptors, C-type lectin receptors, RIG-I like receptors, the inflammasome and the immunoproteasome which makes the genes expressing these proteins interesting candidates for such novel biomarkers.
As it would be of great interest for therapeutic and preventive measures to detect hints predicting an onset of asthma at an early point in life, the analysis of cord blood samples seems to be promising. Not only is it easily available straight after child birth, collection of cord blood is also a non-invasive method of obtaining samples.

Therefore, the following hypotheses were tested in this project:

(1) We hypothesized that the gene expression of genes related to signalling pathways of the innate immune system increases significantly after PHA or LpA stimulation of cord blood mononuclear cells.

(2) We aimed to detect differences in the expression of said genes between the different wheeze-subtypes.

(3) We hypothesized that the regulation of these genes differs between the different wheeze phenotypes and healthy controls.

3. MATERIALS AND METHODS

3.1.Materials

3.1.1. Reagents and chemicals

100bp DNA ladder (500µg/ml)	New England BioLabs, Ipswich, USA
Boric acid	Sigma-Aldrich, Steinheim, Germany
Bromphenol blue	Roth, Karlsruhe, Germany
EDTA	Sigma-Aldrich, Steinheim, Germany
Ethanol 100%	Merck, Darmstadt, Germany
Ethidiumbromide (10mg/ml)	Biorad, Hercules, USA
Glycerol	Sigma-Aldrich, Steinheim,Germany
H2O bidest.	H. Kerndl GmbH, Weißenfeld, Germany
Primers	Life technologies, Invitrogen, Carlsbad, USA
Trizma Base	Sigma-Aldrich, Steinheim, Germany
Water DEPC (0.1 %)	Serva Electrophoresis GmbH, Heidelberg, Germany
Xylene cyanol	Merck, Darmstadt, Germany

3.1.2. Solutions and buffers

5X TBE buffer	54g trizma base 27.5g boric acid 20ml 0.5M EDTA (pH 8.0) Ad 1l H2O bidest.
DNA ladder	10µl 100bp DNA ladder 80µl 0.5x TBE-Buffer 10µl loading dye diluent
Ethidiumbromide [500µg/ml]	100µl ethidiumbromide 1.9 ml H20
Loading dye stock solution	0.25g bromphenol blue 0.25g xylene cyanol 30% glycerol 70ml dH2O
Loading dye diluted solution	5ml loading dye stock solution 13.5ml glycerol 31.5ml dH2O

3.1.3. Reagent systems (Kits)

Sso advanced SYBR green Supermix	Biorad, Hercules, USA
QuantiTect Reverse Transcription Kit	Qiagen, Hilden, Germany

3.1.4. Consumables

96-Well White Shell PCR Plates	BD Biosciences, Heidelberg, Germany
Microseal® ‘B’ seal seals	Biorad, Hercules, USA
Biosphere® filter tips 10µl M 40mm type D	Sarstedt, Nümbrecht, Germany
Biosphere® filter tips 100µl	Sarstedt, Nümbrecht, Germany
SafeGuard Filter tips 100-1000µl	Peqlab, Erlangen, Germany

3.1.5. Laboratory equipment

Centrifuge Perfect SpinP	Peqlab, Erlangen, Germany
Electrophoresis power supply	VWR International, Radnor, USA
Gel iX Imager	Intas Science Images Instruments, Göttingen, Germany
CFX96 Touch™ Real-time PCR Detection System	Biorad, Hercules, USA

3.1.6. Softwares

Biorad CFX Manager 2.1	Biorad, Hercules, USA
EndNote X9	ISI ResearchSoft, Berkeley, USA
Ensembl Genome Browser	http://www.ensembl.org/
National Center for Biotechnology Information	http://www.ncbi.nlm.nih.gov/
R program Version 3.2.2.	http://www.R-project.org/
SPSS version 23	SPSS IBM Inc., Armong, USA
Vector NTI 10 Advance 11.5	Invitrogen, Carlsbad, USA

3.1.7. Primers

Gene	Forward sequence	Reverse sequence
TLR5	GTATTTCTGTGGTCTCTCTGATGCTG	GCTGCGAGGCTAAAAAAGGAG
TLR7	ATGCTGTGTGGTTTGTCTGGTG	ATACCACACATCCCAGAAATAGAGG
RIG-I	GAAGAGAGCAGGATTTGTAAAGCCC	CTGCTCGGACATTGCTGAAGAAG
MDA-5	TTCCGAGAGAAGATGATGTATAAAGC	GCAAAGGAAAGTTATTAGTGATGGG
Mincle	CTACTGACACCATTTCCTGGGCG	TTGCCACTGACCCTCGACAACC
Dectin1	GACTCTCAAAGCAATACCAGGATAGC	TAATCTCCTCCACCAAATACTCACC
Dectin2	TGGCAAAAGGCTGTCTGAACTAC	GCCCCAGAAAATAAGAAAATGACTC
LMP2	AGGCGAGGCGGTGGTGAA	CCTTCACGTTGGTCCCAGCC
LMP7	CCACCACGCTCGCCTTCAA	TCCTGAGAGCCGAGTCCCATG
NLRP3	AAAGCAAAAAGAGATGAGCCGAAG	AGTCGTGTGTAGCGTTTGTTGAGG
Casp1	CGCTTTCTGCTCTTCCACACC	CGCTCTACCATCTGGCTGCTC
IL1R1	GCATCCTACACATACTTGGGCAAG	GTAATTGATGAAGATGACCCAGTGCT
18S	AGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC

3.2.Study population

3.2.1. PAULINA

In the **P**ediatric **A**lliance for **U**nselected **L**ongitudinal **I**nvestigation of **N**eonates for **A**llergies (PAULINA) study [74], cord blood samples from newborns of the Munich metropolitan area, Germany, were collected. Atopic and non-atopic mothers were recruited in the last trimester of pregnancy.
The study has been approved by the Bavarian Ethical Board, LMU Munich, Germany.
In collaboration with the delivery room nurses of the University gynecology hospital (Maistrasse, LMU) n=190 mothers were recruited between October 2004 and September 2007. Inclusion criteria were an uncomplicated pregnancy and healthy neonates and exclusion criteria contained preterm delivery, perinatal infections, maternal use of antibiotics during the last trimester of pregnancy and chronic diseases of the mother.
Two groups were recruited: neonates with an atopic mother and neonates with a non-atopic mother. Maternal atopy was defined as having received a doctor's diagnosis of asthma and/or allergic rhinitis and/or atopic dermatitis.
Cord blood samples from the neonates along with blood samples from the mothers were collected. Sample processing was performed within 24h in our laboratory.
Cord blood mononuclear cells (CBMCs) were isolated and exposed to different innate and adaptive immune response related stimuli and mitogens in order to find out whether the immune system of newborns from atopic mothers reacts different to those stimuli than newborns from non-atopic mothers.
In maternal blood, total IgE along with specific IgE (Immunoblot assay) was measured. A positive specific IgE was defined as ≥0.35 IU/mL to one or more common allergens from a panel of 20 allergens including plants, animals, foods, latex and house dust mite.

3.2.2. PAULCHEN

In the PAULCHEN study (Prospective Cord Blood Study in Rural Southern Germany) [3], 91 pregnant mothers from rural southern Germany were recruited from September 2005 to December 2008.
Approval was obtained from the Bavarian Ethical Board, LMU Munich, Germany.
In collaboration with the obstetric clinic (Asklepios Clinic) Bad Tölz, cord blood samples were collected and processed within 24h in our laboratory.
Inclusion and exclusion criteria were equal to the PAULINA study (see above).
In contrast to the PAULINA study, the following groups were defined: the farming group was defined as the mother having lived and/or worked on a farm during pregnancy whereas the non-farming group was defined as the mother having lived in rural environment during pregnancy. In order to increase the total sample size, a selected number of children from the PAULCHEN study was included in this project. Only non-farming children were selected as they have no general protection from allergy and therefore are easily comparable with children from the PAULINA study.
Sample material and work-flow were equal to the PAULINA study (see above).

3.2.3. Follow up

At the age of 3 years as well as at the age of 6 years a follow-up study was performed. For this purpose, the parents completed a detailed questionnaire including information about both present and past symptoms. Special focus has been put on the follow up of wheeze symptoms, airway inflammation and allergy symptoms. The follow up was performed in both study cohorts and is still on-going at age of 10 years currently.
Those data were used for the phenotype definition of the children.

Table 6: comparison of PAULINA and PAULCHEN study

	PAULINA	**PAULCHEN**
Focus	Difference between the newborn's immune responses to different stimuli in correlation with the mother's atopy status	Difference between the newborn's immune responses to different stimuli in correlation with the mother's farming status during pregnancy In this project only non-farming children were included
Recruitment time span	October 2004 – September 2007	September 2005 – December 2008
Total sample size	n= 190	n=93
Inclusion and exclusion criteria	Inclusion: uncomplicated pregnancy and healthy neonate Exclusion: preterm delivery, perinatal infections, maternal use of antibiotics during the last trimester of pregnancy, chronic diseases of the mother	
Follow- up	Age 3 years, age 6 years	Age 3 years, age 6 years

Blood sample collection, processing and stimulation was identical for both study populations allowing the joint data analysis performed in this project.

3.3. Declaration of my contribution

Due to the longitudinal character of the PAULINA/PAULCHEN studies, some of the laboratory work took place prior to this project. The recruitment of patients, from 2004 to 2008, the cell stimulation directly following the blood withdrawal and the RNA extraction were performed by group members of the AG Schaub.
As this project focused on finding potential new biomarkers for the prediction of childhood wheeze, I designed and selected the primers for this project, performed the synthesis of cDNA, the following quantitative real time PCR, gel electrophoresis and

the quality control. I also checked the data analysis myself and performed the basic statistical analysis. The more complex statistical analysis has been supervised.

3.4. Blood withdrawal

All participating families were informed about the study by a physician and gave their written consent.
Blood withdrawal of the umbilical cord blood took place directly after delivery and was performed by a midwife or obstetrician. 30-40ml of blood were taken and treated with Liquemin for anticoagulation. Additionally, 1ml of blood was taken and stored in an EDTA tube at -80°C.
Furthermore, 4.9 ml serum from the mother were obtained from a peripheral vein.

3.4.1. Detection of maternal cells in the cord blood samples by karyotyping

In order to ensure no relevant contamination of the cord blood cells with maternal cells, some male samples were tested for potential contamination. For this purpose, isolated CBMCs were incubated with Colcemid (Invitrogen, Karlsruhe), a microtubule-depolymerizing drug, for 30 min. The resulting precipitate was centrifuged and resuspended in 0.54% potassium chloride and again incubated for 25min at 37°C. The cells were then fixed in methanol with the help of a 33% glacial acetic acid solution and put on slides.
The X and Y chromosomes were stained with a dichromatic alpha-Satellite Kit (Rainbow Scientific, Banbury, UK), thus allowing differentiation between maternal cells with a XX karyotype and fetal cells with a male XY karyotype.
There was no relevant contamination of maternal cells detected in the CBMCs. This experiment took place in the beginning of the study in order to ensure good sample quality for all following projects including this one [75].

3.4.2. Isolation of CBMCs

Within 24h after blood withdrawal in the delivery room, CBMC isolation was performed by a group member of the AG Schaub.
For CBMC isolation the blood was diluted 2:1 with PBS and cells were isolated via Ficoll (GE Healthcare, Piscataway, USA) density centrifugation. Ficoll separates cells along their density gradient and thereby allows separation of mononuclear cells from erythrocytes.
After centrifugation (30min, 20°C, 1400rpm, without brake), the mononuclear cell layer was harvested from the tube, diluted up to a volume of 10 ml with the culture medium RPMI (Gibco, Carlsbad, USA), centrifuged for 10 min, 2400 rpm and the supernatant was discarded. Cell number was determined by counting in the Neubauer counting chamber under the microscope.

After a second centrifugation step, the precipitate containing the cells was diluted in RPMI containing 10% human serum to a cell concentration of $5x10^6$ cells/ ml for cell culture.

3.4.3. Cell culture and stimulation

The isolated CBMCs were stimulated with different substances triggering an immune response. Two of those stimuli and the control condition without any stimulation were analyzed in this project.
One stimulus is Phytohaemagglutinin (PHA) with a concentration of 5µg/ml that acts as an unspecific activator of T cells. The other is Lipid A (LpA), the lipid component of Lipopolysaccharides which is an endotoxin from gram-negative bacteria, with a concentration of 0.1µg/ml that is known to trigger T_H1 response.
After stimulation with either 3µl PHA or LPA, the cells, among with unstimulated cells (Media), were incubated for 72h at 37°C temperature and a CO_2 concentration of 5%.
Subsequently, the cells were manually picked and supernatant was removed and kept at -20°C for cytokine measurements. The resulting cells were then resuspended in PBS, the supernatant was spun down and discarded and the cells were infused with 1ml TRIzol (Invitrogen, Karlsruhe, Germany) leading to the lysis of cell membrane which is necessary for RNA extraction and stabilization. The samples were then stored at -80°C.

3.5. Determination of gene expression

3.5.1. RNA extraction

For RNA extraction, thawed cell pellets were resuspended in 0.2 ml chloroform. After 10 minutes incubation the mix was centrifuged for 15min at 4°C and 1200rpm. 0.5 ml 100% isopropanol and 1µl glycogen were added after removal of the aqueous phase. Then, the mix was centrifuged again, the supernatant was removed and 75 percent ethanol was added followed by one more centrifugation. Afterwards, the RNA precipitate was dried on a heating block at 42°C for 10-30 min. The resulting samples were resuspended in RNAse-free water, incubated at 55-60°C and then either stored at -80°C or directly used for further analysis.
Sample processing down to RNA extraction took place prior to the start of this project.

3.5.2. Synthesis of cDNA

cDNA was synthesized following the Qiagen-Kit (QuantiTect) instructions which includes the elimination of possible genomic DNA (gDNA) contamination by adding a gDNA wipeout buffer.
After determining the RNA concentration by photometric measurements with *nanodrop* (Peqlab by VWR, Erlangen), 1µg RNA was used for processing cDNA.
The RNA was treated with 2 µl wipeout buffer, filled to 14 µl with RNA-free water and heated for 2 minutes at 42°C in the *RNA-Cycler*.

Reverse-transcriptase mix (1 µl reverse transcriptase, 4 µl QRT buffer and 1 µl primer mix) was added and then incubated in the cycler for 15 minutes at 42°C.
To inactivate the reverse transcriptase, the sample was heated to 95°C for 3 minutes. The resulting cDNA was consequently solved in 20 µl, which lead to a final cDNA concentration of 50ng/µl, as 1 µl RNA was used for transcription.
The cDNA was stored at -20°C and then used for quantitative real-time PCR.

3.5.3. Primer design

Primers are small nucleotide sequences that are specifically designed to bind to a certain gene segment. They mark the start point for the polymerase that then amplifies the gene sequence. Primers for the genes of interest were designed with the help of *"Vector NTI"* program (Invitrogen, Karlsruhe) and ordered from Invitrogen.
Primer design was based upon DNA sequences provided by the genome database *"Ensemble"*, a joint project between the European Bioinformatics Institute (EBI), the European Molecular Biology Laboratory (EMBL) and the Wellcome Trust Sanger Institute (WTSI). Sequences were looked up in the *"Ensemble"* database and then fed into the *"Vector NTI"* program.
In order to find a matching primer pair, which consists of a forward and a reverse primer, the following rules were obeyed:

- Primer length should be between 18 and 27 base pairs
- At 3´end there should be at least one guanine or cytosine
- Melting temperature of the primer should be between 54°C and 65°C with a temperature difference no bigger than 0.5°C between forward and reverse primer
- Primers have to be located behind the ATG sequence of the gene as the processed RNA starts at this point
- Energy to build dimers or hair pins should be lower than ±2 kcal/mol in order to assure good annealing efficiency
- Percentage of guanine and cytosine should be between 40-60% with no more than 10% difference between forward and reverse primer
- If possible, forward and reverse primer should be located on different exons with as large introns as possible in between to avoid amplification of gDNA residues
- The resulting PCR product should be around 200 base pairs long

Delivered primers were diluted with DEPC-water into a 1mM stock. Afterwards, a 1 µM dilution was made via an intermediate step of a 0.1 mM dilution. This 1 µM dilution contained both forward and reverse primers, was stored at +4°C and used for qRT PCR.
The diluted primers were then tested for quality. In a first step, using a test sample, the general primer properties were tested with the focus on amplification and the corresponding melting curves.
Primers holding up to those criteria were then tested for specificity. Therefore, they were tested with both gDNA and RNA and were analyzed for unwanted amplifications.
Primers for genes with only one exon sometimes bound to gDNA. By means of the melting curve analysis we were never the less able to differentiate between cDNA

amplification and unwanted gDNA amplification. Additionally, as we added the gDNA wipeout buffer during RNA isolation, contamination with gDNA was highly unlikely in the used samples.

3.5.4. Principle of PCR

The polymerase chain reaction (PCR) is a technology used to specifically amplify DNA. Even smallest amounts of DNA (down to a single copy) are sufficient. PCR mimics the natural process of DNA amplification taking place e.g. in human cells.
In a first step, the DNA double helix is decomposed by heat denaturation at 96°C, yielding two single-stranded DNA molecules. This step is called initialization. In addition to the decomposition of DNA, the high temperature activates the involved polymerase.
Starting with the second cycle, the following steps are repeated in each PCR cycle:

1. Denaturation:
   In the beginning, the newly formed DNA is decomposed into single strands by heating it up to 95°C.
2. Annealing:
   At 62.5°C, primers anneal to the 5‘-3‘– end of the gene section to be amplified. This step takes about 30 seconds. Choosing the right temperature is crucial as an incorrect temperature my lead to non-specific amplifications. The listed temperatures refer to the specific conditions in this project.
3. Elongation:
   The thermostable Taq Polymerase elongates the annealed primers at 72°C. The desoxynucleotide triphosphates are part of the added Mastermix. Elongation continues until either the Taq polymerase reaches the end of the strand or the process is interrupted by a new cycle of heating.

Theoretically, amplification is an exponential process as can be seen in the following equation:

$$N_n = N_0 \times 2^n$$

Equation 1: exponential increase of cDNA with
N_n = amount of cDNA after n cycles
N_0 = amount of cDNA in the beginning (prior to first amplification)
n = amount of cycles

For this to be true, efficiency of Taq polymerase would have to be 100% leading to a reduplication of cDNA in each cycle. As this doesn’t apply in reality, every analysis of PCR data should contain a correction for efficiency. Therfore quantiatative real-time PRC was used in this project.

3.5.5. Quantitative real-time PCR

In contrast to conventional PCR, quantitative real-time PCR (qRT PCR) can not only amplify DNA sections but also provides information about the original amount of cDNA in the sample. As cDNA that resulted from the mRNA of the cord blood cells was used in this project, this method allows investigation of the gene expression on RNA level in the unstimulated and stimulated CBMCs.
Quantitative analysis in qRT PCR is mediated through a fluorescence marker that binds to the amplified gene segment. In this project the fluorescent dye *SYBR-Green* (Biorad, Hercules, USA) was used which intercalates into double-stranded DNA and then transmits a fluorescence signal.
Reaching a certain amount of DNA product, the fluorescence signal exceeds the so-called threshold. The earlier this happens the higher the initial RNA concentration in the sample has been.
SYBR-Green is a highly sensitive measuring system but it does not only intercalate into DNA but also with primer dimers and byproducts of PCR. To certify specificity, analysis of the melting curves is indispensable.

3.5.6. Pipetting scheme

	1	2	3	4	5	6	7	8	9	10	11	12
A	Mincle M	TLR7 M	TLR5 M	Dectin1 M	Dectin2 M	RIG-I M	MDA-5 M	NLRP3 M	Casp1 M	LMP2 M	LMP7 M	IL-1R1 M
B	Mincle M	TLR7 M	TLR5 M	Dectin1 M	Dectin2 M	RIG-I M	MDA-5 M	NLRP3 M	Casp1 M	LMP2 M	LMP7 M	IL-1R1 M
C	Mincle PHA	TLR7 PHA	TLR5 PHA	Dectin1 PHA	Dectin2 PHA	RIG-I PHA	MDA-5 PHA	NLRP3 PHA	Casp1 PHA	LMP2 PHA	LMP7 PHA	IL-1R1 PHA
D	Mincle PHA	TLR7 PHA	TLR5 PHA	Dectin1 PHA	Dectin2 PHA	RIG-I PHA	MDA-5 PHA	NLRP3 PHA	Casp1 PHA	LMP2 PHA	LMP7 PHA	IL-1R1 PHA
E	Mincle LpA	TLR7 LpA	TLR5 LpA	Dectin1 LpA	Dectin2 LpA	RIG-I LpA	MDA-5 LpA	NLRP3 LpA	Casp1 LpA	LMP2 LpA	LMP7 LpA	IL-1R1 LpA
F	Mincle LpA	TLR7 LpA	TLR5 LpA	Dectin1 LpA	Dectin2 LpA	RIG-I LpA	MDA-5 LpA	NLRP3 LpA	Casp1 LpA	LMP2 LpA	LMP7 LpA	IL-1R1 LpA
G	18S M	18S PHA	18S LpA	B2mic M	B2mic LpA	Mincle NTC	TLR7 NTC	TLR5 NTC	Dectin1 NTC	Dectin2 NTC	RIG-I NTC	MDA-5 NTC
H	18S M	18S PHA	18S LpA	B2mic PHA		NLRP3 NTC	Casp1 NTC	LMP2 NTC	LMP7 NTC	IL-1R1 NTC	18S NTC	B2mic NTC

Figure 8: pipetting scheme
Lines A to F represent the different stimuli (PHA, LpA) along with the unstimulated cells (M) in duplicates and column 1-12 represent the different primers. Wells G 1-5 and H 1-4 contain the housekeeping genes and G and H 6-12 the non-template controls (NTCs). Into every well, 5µl SSo advanced SYBR green Supermix were pipetted, followed by 1.8µl cDNA mix for the stimuli and 1.8µl RNAse-free water for the NTCs. Then, 3.2µl primers were added into every well leading to a total volume of 10µl/well.

Pipetting was performed quickly and on ice according to the pipetting scheme in Figure 8.

In addition to the selected genes for this project, 18S and β2mic, so-called *housekeeping genes*, were applied and used as reference genes. *Housekeeping genes* are genes that are constitutively expressed in a cell reflecting the base-line cell activity.
After pipetting, the qRT PCR plates were covered with a transparent film and centrifuged at 2500 rpm for 15 seconds to eliminate possible air bubbles. Immediately after centrifugation, the plates were put into the *iCycler* (Biorad) and qRT PCR was performed.

3.5.7. *iCyler* protocol

Cycle 1: (1x)	95,0°C	2 minutes (=initialization)
cycle 2: (40x) step 1: step 2+3:	 95,0°C 62,5°C	 20 seconds (=denaturation) 30 seconds (=annealing+ elongation)
Cycle 3: (1x)	72,0°C	2 minutes (=Elongation)
Cycle 4: (1x)	95,0°C	30 seconds
Cycle 5: (1x)	55,0°C	30 seconds
Cycle 6: (80x)	55,0°C	10 seconds
Cycle7: (1x)	20,0 °C	HOLD

Table 7: *iCycler* protocol

3.5.8. Gel electrophoresis

Gel electrophoresis is the separation of molecules and their fragments by using their different moving properties through an electric field. Positively charged molecules move towards the cathode whereas negatively charged move towards the anode. The smaller the molecule the faster and further it moves through the gel leading to a separation based on the different molecule sizes.
This process is modulated by the concentration of the gel. A higher concentration of agarose results in a more close-mesh gel and a more precise separation of smaller fragments (down to 50 base pairs). Nucleic acids are negatively charged because of their sugar-phosphate back bone and therefore move towards the anode.
Gel electrophoresis was used in this project to separate and assess the amplification products of qRT PCR.
In order to make the 3 percent agarose gel, 6g agarose together with 200ml of 0.5-fold buffer (900 ml aqua bidest+100 ml 5-fold TBE) were dissolved in a heat-resistant bulb and then heated until the solution was clear. After a short cooling time, 70 µl ethidium bromide were added. Ethidium bromide intercalates into DNA making nucleic acids visible under UV light. Afterwards, the gel was poured into a chamber, combs were stuck into it and then the gel cooled down for 30 minutes (see Figure 9). By pulling the combs out of the cold gel, they formed small pockets.

4 µl *loading dye* were added to both PCR products and NTCs. *Loading dye* contains a very high percentage of glycerin and thereby weighs down the samples keeping them inside the pockets. PCR products, the NTCs and the so-called *ladder*, a reference standard containing DNA fragments of known length that provides a scale to estimate the size of the PCR products, were pipetted into the gel pockets.
Gel electrophoresis was performed by applying electrical current at 120 V voltage and 400mA amperage for 40 min.
The gel was analyzed under UV light and a picture of every gel was taken.

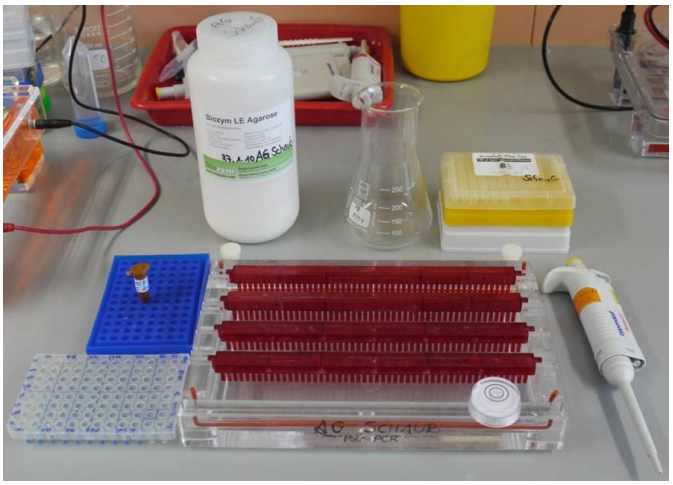


Figure 9: poured gel with inserted combs, photo provided by E. Klucker

3.5.9. Quality control

3.5.9.1. Primer selection

In order to assure PCR with ΔC_T values of high quality, primers were chosen based upon their melting curve and their specificity for cDNA.

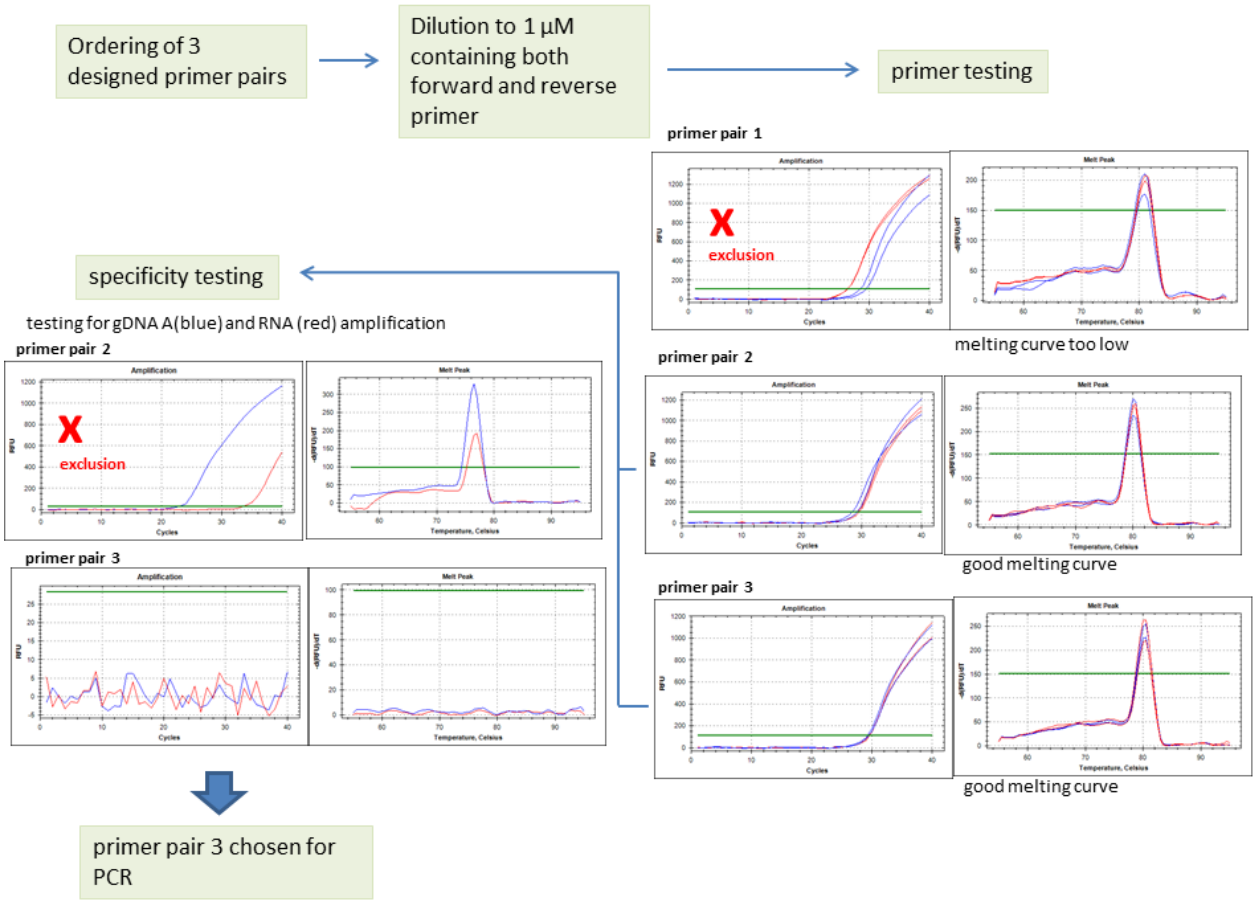


Figure 10: work flow for primer selection

3.5.9.2. Melting curve analysis

The melting curve generated by the *iCycler* (see 3.5.10) was checked for unwanted or unspecific amplification of e.g. primer dimers or cDNA contamination. An ideal melting curve features a narrow and high peak and is as similar as possible between both duplicates.

In contrast, primer dimers have a flat and wide melting curve which makes it possible to differentiate between non-specific amplifications and the wanted gene amplification (see Figure 11).

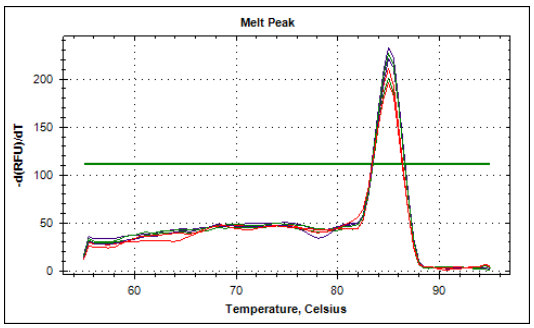
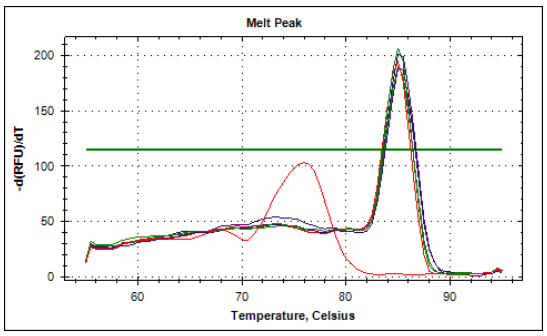


Figure 11: a) example of melting curve from a wanted gene amplification with a narrow, high peak



b) example of melting curve from an unwanted amplification with a flat, wide peak

ΔC_T values featuring an unspecific melting curve or with a difference greater than 1 PCR cycle between the duplicates were repeated and, if they still couldn't hold up to quality criteria, were excluded.

3.5.9.3. Quality control by gel electrophoresis

Additionally, the PCR quality was checked for unspecific amplification by performed gel electrophoresis. The gel was searched for double bands indicating said unspecific amplification and was checked for aberrant bands by comparison of the expected size of PCR bands (around 200 bp) with the added *ladder*.

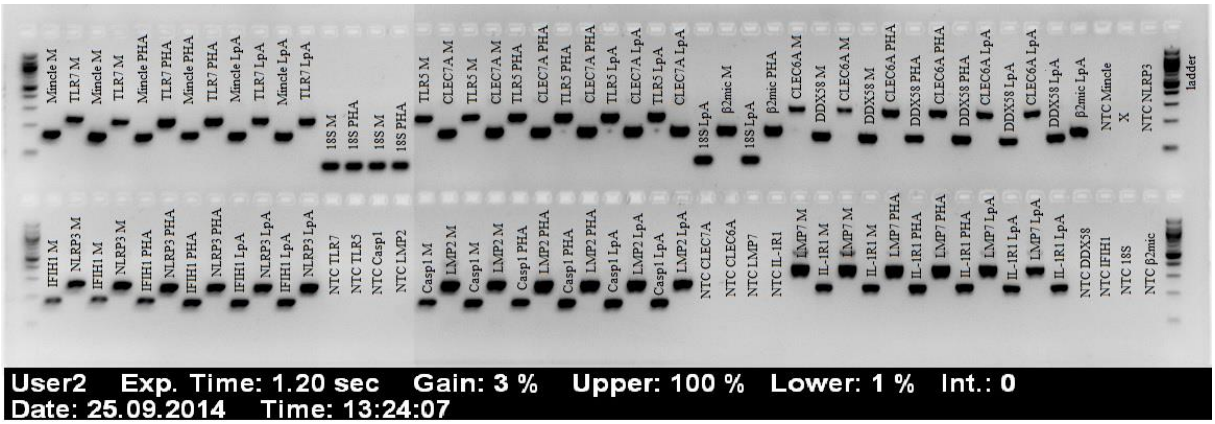


Figure 12: gel with bands labeling including genes of interest, stimuli and NTCs and ladder.

If the gel electrophoresis showed any unwanted amplification, the PCR was repeated.

3.5.10.Data analysis

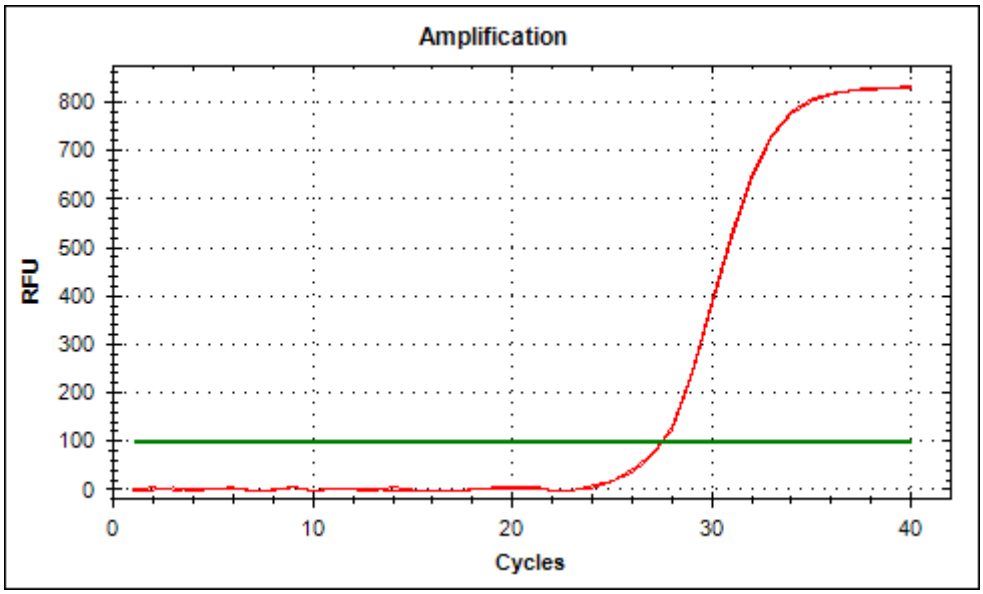


Figure 13: cDNA amplification in *iCycler* program. Abscissa shows the number of PCR cycles and ordinate the intensity of fluorescence signal. The green line represents the *threshold*. The interception with the *threshold* is called C_T value and is used for analysis. Red line shows rise of the fluorescence signal. In this project, duplicates were produced.

The *axis of abscissae* shows the number of PCR cycles and the *axis of ordinate* the intensity of the fluorescence signal.
Green fluorescent signal treshold is marked by the green line (*ordinate intercept 100*) and is determined by the *iCycler* program. It can also be chosen manually and should be located at the beginning of the exponential phase of the curve progression.
After a certain amount of amplifications, the fluorescence signal rises above the *threshold* and starts growing exponentially. This value is called C_T value (*threshold cycle*) and is used for analysis. A high gene expression results in a low C_T value caused by the fact that a high concentration of cDNA leads to an early increase of the fluorescence signal.
Curve progression ends in the plateau phase were optimal conditions for PCR do no longer apply and amplification ends. As mentioned earlier, for quality control of PCR a melting curve analysis should be performed.
The *iCycler* generates the melting curve by continuously measuring with rising temperature from 55°C up to 95°C in 0.5°C steps. Every PCR product has its specific denaturation temperature leading to a measurable decrease in the fluorescence signal. As mentioned, the ideal melting curve features a narrow and high peak and is as similar as possible between both duplicates.

3.5.11.Statistical analysis

Statistical analysis was performed using *Excel* program and SPSS *Statistics* program Version 23 and *R* program (R Core Team (2016). R: A language and environment for sta tistical computing. R Foundation for Statistical Computing, Vienna, Austria, https://www.R-project.org/).

First of all, the quality proofed C_T values were entered into *Excel*. In this step values of poor quality were excluded from analysis.
Some genes were not detectable due to technical limitation. The C_T value of those genes was defined as 40 which corresponds to the number of the last performed PCR cycle in the protocol.
In a second step, to include the different base-line gene expression of every sample into calculation, difference between expression of the particular gene and the housekeeping gene 18S was calculated resulting in so-called ΔC_T values.

ΔC_T= C_T value gene$_x$ – C_T value 18S

The expression of 18S was, as expected, higher than the expression of the genes of interest leading to positive ΔCT values.

To define how the different stimuli affect the gene expression, difference between ΔCT values of the stimulated samples and the ΔCT of the unstimulated samples (media) was calculated. The resulting $\Delta\Delta C_T$ value can either be positive as a correlate of upregulation or negative as a correlate of downregulation.

$\Delta\Delta C_T$= ΔC_T value gene$_x$ media - ΔC_T value gene$_x$ stimulated

One parametric model contributing to the characteristics of censoring problem within the gene expression data is the so-called *tobit* method.

Applied to the gene expression data, only values between 1 and 40 (meaning between the first and the last cycle of qRT PCR) are detectable due to technical limitation. If the gene amplification is beyond this value, the expression of the gene is too low to be determined.

In an equation:

$$y = \begin{cases} y^* & y < 40 \\ 40 & y \geq 40 \end{cases}$$

Equation 2: censoring mechanism
y= theoretical gene expression; y^*= measured gene expression

To calculate the mean value of y (not y^*), the *tobit* model is applied. Furthermore, this model allows including additional co-variables, in this case the different phenotypes. Therefore, a comparison between the different phenotypes can be performed as well as an adjustment for possible confounders.
To analyze whether the stimulation conditions had an effect on the gene expression, Wilcoxon sign ranked test was performed.
To test for difference of the gene expression between the different wheeze phenotypes, Mann-Whitney-U test was performed.
Statistical significance was defined as a p-value < 0.05.
For genes that showed significant differential expression between the phenotypes, the effect of confounding study characteristics was assessed in a stratified analysis.

4. RESULTS

4.1. Phenotype definition

Phenotypes were defined on the basis of questionnaires completed by the parents at both age 3 years and age 6 years of the children (see page 106).
Table 8 exemplifies how the different phenotypes were defined. Shortly, healthy controls were defined by no symptoms at any age. Multitrigger wheeze was defined by wheezing caused by multiple triggers (e.g. effort, cold, house dust, animal contact, pollen, others). Additionally, some children featured a positive allergy test and suffered from rhinoconjunctivitis. Children presenting with wheeze associated to viral infection, were divided into two groups: early viral wheeze and persistent or late onset viral wheeze. Patients presenting with viral wheeze only within the first 3 years of life were defined as early viral wheeze. Persistent or late onset viral wheeze was defined by symptoms at age 6 years during an acute infection in combination with age 3 years symptoms. Consistency of information was checked using all 3 questionnaires (birth, age 3 years and age 6 years).

Table 8: phenotype characteristics based upon the 6-year questionnaire

Phenotype	*Question 1:* *'Has your child ever had wheezing?'*	*Question 2* *'Has your child wheezed in the past 3 years?'*	*Additional questions*
Healthy control	no	no	*'Has your child been prescribed medication for wheezing or shortness of breath in the last 3 years?'*(Question 10) : no *'Has your child been diagnosed with obstructive or spastic bronchitis or asthmatic bronchitis?'* (Question 34): no
Multitrigger wheeze	yes	yes	'*What triggers the wheezing?'*(Question 6): at least two different triggers *'How often does your child wheeze when they are not having an acute infection?'*(Question 7): at least once a month
Early viral wheeze	yes	no	*'Is your child completely symptom-free between the wheezing episodes?'*(Question 8): yes *'How often does your child wheeze when they're not having an acute infection?'* (Question 7): never

Persistent or late onset viral wheeze	yes	yes	*'Is your child completely symptom-free between the wheezing episodes?'*(Question 8): yes *'How often does your child wheeze when they're not having an acute infection?'* (Question 7): never

In a second step, the defined phenotypes persistent or late onset viral wheeze and multitrigger wheeze were characterized more closely by considering the temporal aspect. This means, the questionnaires were analyzed independently from each other. If the criteria for multitrigger wheeze applied at both ages, the multitrigger wheeze was defined as persistent. If the child either showed no symptoms or presented as a viral wheezer at age 3 but developed multitrigger-like symptoms at age 6, multitrigger wheeze was defined as late onset. In this step, the group of persistent and late onset viral wheeze was divided into either persistent viral wheeze or late onset viral wheeze by including the age 3 years questionnaire.
Figure 14 summarizes the phenotype characteristics.

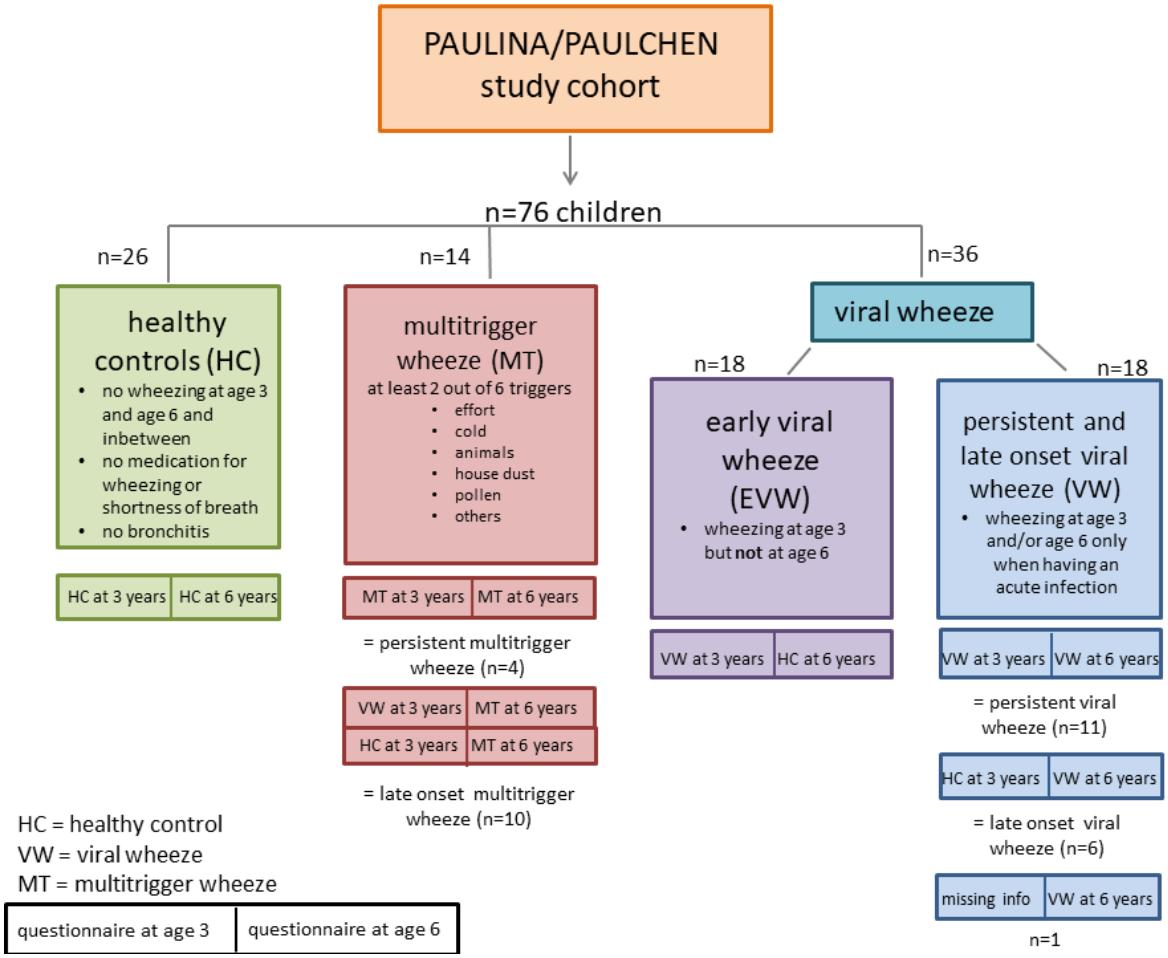


Figure 14: phenotype characteristics

4.2.Study characteristics

Table 9 summarizes the study characteristics of the PAULINA/PAULCHEN sub cohort (n=76 children) that was analyzed in this project. The selection was based upon a case-control design based on future phenotype definition (matching range 1:1 to 1:2). Statistical significance of differences between the phenotypes was analyzed using Kruskal-Wallis test for the continuous variables (birth weight, maternal age and maternal education) and Wilcoxon signed-rang for the categorical variables. The tests were performed by group comparison of all groups. The only statistically significant differences were obtained for maternal asthma when comparing healthy controls with children with viral wheeze and for maternal education when comparing children with multitrigger wheeze with children with early viral wheeze.

Table 9: Study characteristics for the samples analyzed in this project (n total=76).

		Healthy controls (HC) (n=26)	**Multitrigger wheeze (MT)** (n=14)	**Early viral wheeze (EVW)** (n=18)	**Viral wheeze (VW)** (n=18)	p-value
Male sex		10 (38.5%)	8 (57.1%)	12 (66.7%)	12 (66.7%)	n.s.[b]
Birth weight in grams		3510.0 (3146.25;3748.75)	3737.5 (3590.0;3965.0)	3660.0 (3342.5;3837.5)	3570.0 (3402.5;3797.5)	n.s.[a]
Maternal age at birth in years		33.5 (30.75;36.25)	32.5 (28.5;34.75)	35 (32.5;36.0)	32.0 (31.0;34.0)	n.s.[a]
Maternal asthma		0 (0%)	2 (14.3%)	3 (16.7%)	4 (22.2%)	HC vs VW **0.02[b]**
Maternal atopy		7 (26.9%)	8 (57.1%)	7 (38.9%)	10 (55.6%)	n.s.[b]
Maternal smoking	*during pregnancy*	1 (3.8%)	0 (0%)	1 (5.6%)	1 (5.6%)	n.s.[b]
	stopped in pregnancy	3 (11.5%)	1 (7.1%)	0 (0%)	3 (16.7%)	n.s.[b]
	stopped before pregnancy	0 (0%)	1 (7.1%)	0 (0%)	0 (0%)	n.s.[b]
	never smoked	22 (84.6%)	12 (85.7%)	17 (94.4%)	14 (77.8%)	n.s.[b]
Maternal education (school years)		16.0 (10.0;16.0)	13.0 (10.0;16.0)	16.0 (16.0;16.0)	16.0 (13.0;16.0)	MT vs. EVW **0.01[a]**
Paternal atopy		10 (30.8%)	8 (57.1%)	9 (50.0%)	6 (33.3%)	n.s.[b]

a= Kruskal-Wallis test

b= Wilcoxon signed-rang test

4.2.1. Stratification for maternal asthma and maternal school years

In a second step, association of gene expression and both maternal asthma and maternal school education were tested in order to identify potential confounding.
There was no significant association between gene expression and maternal asthma detectable.
For maternal school education three single associations were significant (p-value <0.05): LMP2, LMP7 and NLRP3 (all after PHA stimulation). Children of mothers with more school years showed a slightly lower gene expression. Therefore, these genes were analyzed stratified for maternal school years.
For this analysis, the different wheeze phenotypes were divided in subgroups according to maternal school years leading to children with either 9, 10, 13 or 16 years of maternal education. In a second step, group comparisons of significant findings were recalculated within the stratified subgroups.
Overall, the found upregulation of gene expression in children with multitrigger wheeze remained unchanged. Due to the smaller sample size in the subgroups, said upregulation was not as significant as in the unstratified analysis.
For the four-group phenotype analysis, there was a tendency towards a lower gene expression of LMP2 and LMP7 in children with multitrigger wheeze and 16 years of maternal education compared with children with less maternal education years. However, this was not statistically significant.
In the more detailed phenotype analysis, there was a tendency towards lower gene expression of NLRP3 in children with late onset multitrigger wheeze and 16 years of maternal education. However, these findings were not statistically significant.
The affected findings and associated results after stratifying for maternal school years are listed in the attachments (see page 120).

4.3. Gene expression

In the following, the *y-axis* is scaled reversely. Therefore, values higher up the *y-axis* represent a lower ΔCT value meaning a higher gene expression.

4.3.1. Technical exclusion

Based on previous publications, we have shown that a group size of 14-16 children is sufficient for significant results [3, 74]. In a nested case-control study design, a 1:1 to 1:2 matching was planned. Thus, of the total amount of n=200 children in the PAULINA cohort, n= 69 children were analyzed in this project. Additionally, n=7 children from the PAULCHEN multitrigger sub group were analyzed in order to reach an adequate sample size.
One limiting factor for sample selection was the amount of cDNA available resulting in some samples with cDNA lacking for one or more stimuli.
Furthermore, some ΔC_T values had to be excluded from analysis as they couldn't hold up to strict quality criteria. Table 10 shows the number and percentage of excluded samples for each gene.

Table 10: number and percentage of excluded sample per gene and stimulus

Gene	*Media (%)*	*PHA(%)*	*LpA(%)*
TLR5	1(1.3%)	6 (7.9%)	6 (7.9%)
TLR7	1(1.3%)	2 (2.6%)	6 (7.9%)
RIG-I (DDX58)	0 (0%)	2 (2.6%)	5 (6.6%)
IFIH1 (MDA-5)	1(1.3%)	1 (1.3%)	4 (5.3%)
Mincle	2 (2.6%)	4 (5.3%)	4 (5.3%)
Dectin1	2 (2.6%)	2 (2.6%)	4 (5.3%)
Dectin2	5 (6.6%)	5 (6.6%)	6 (7.9%)
LMP2	0 (0%)	1 (1.3%)	4 (5.3%)
LMP7	0 (0%)	3 (3.9%)	7 (9.2%)
NLRP3	0 (0%)	3 (3.9%)	4 (5.3%)
Casp1	1(1.3%)	1 (1.3%)	4 (5.3%)
IL-1R1	1(1.3%)	1 (1.3%)	5 (6.6%)

4.3.2. Detection levels of gene expression

Due to technical limitations, some genes were not detectable. Therefore, these genes were set at a ΔC_T value of 40 which corresponds to the last performed PCR cycle (see 3.5.11). Table 11 shows the percentage of uncensored data for every gene within those that were included for analysis. Only Dectin2 featured less than 80% uncensored data.

Table 11: percentage of uncensored samples for each gene and stimulus within included data

Gene	*Media %*	*PHA %*	*LpA(%)*
TLR5	88.0	80.0	91.4
TLR7	81.2	85.1	92.9
RIG-I (DDX58)	88.2	93.2	98.6
IFIH1 (MDA-5)	86.7	88.0	95.8
Mincle	89.2	93.1	98.6
Dectin1	94.6	91.9	98.6
Dectin2	43.7	63.4	87.1
LMP2	96.1	100.0	100.0
LMP7	88.1	95.9	98.6
NLRP3	90.8	87.7	96.6
Casp1	90.7	93.3	98.6
IL-1R1	82.7	88.0	94.4

4.3.3. Gene- gene correlations

The calculation of gene-gene correlations was performed using pair-wise-complete spearman correlations and showed a positive correlation with an average correlation coefficient around 0.6.

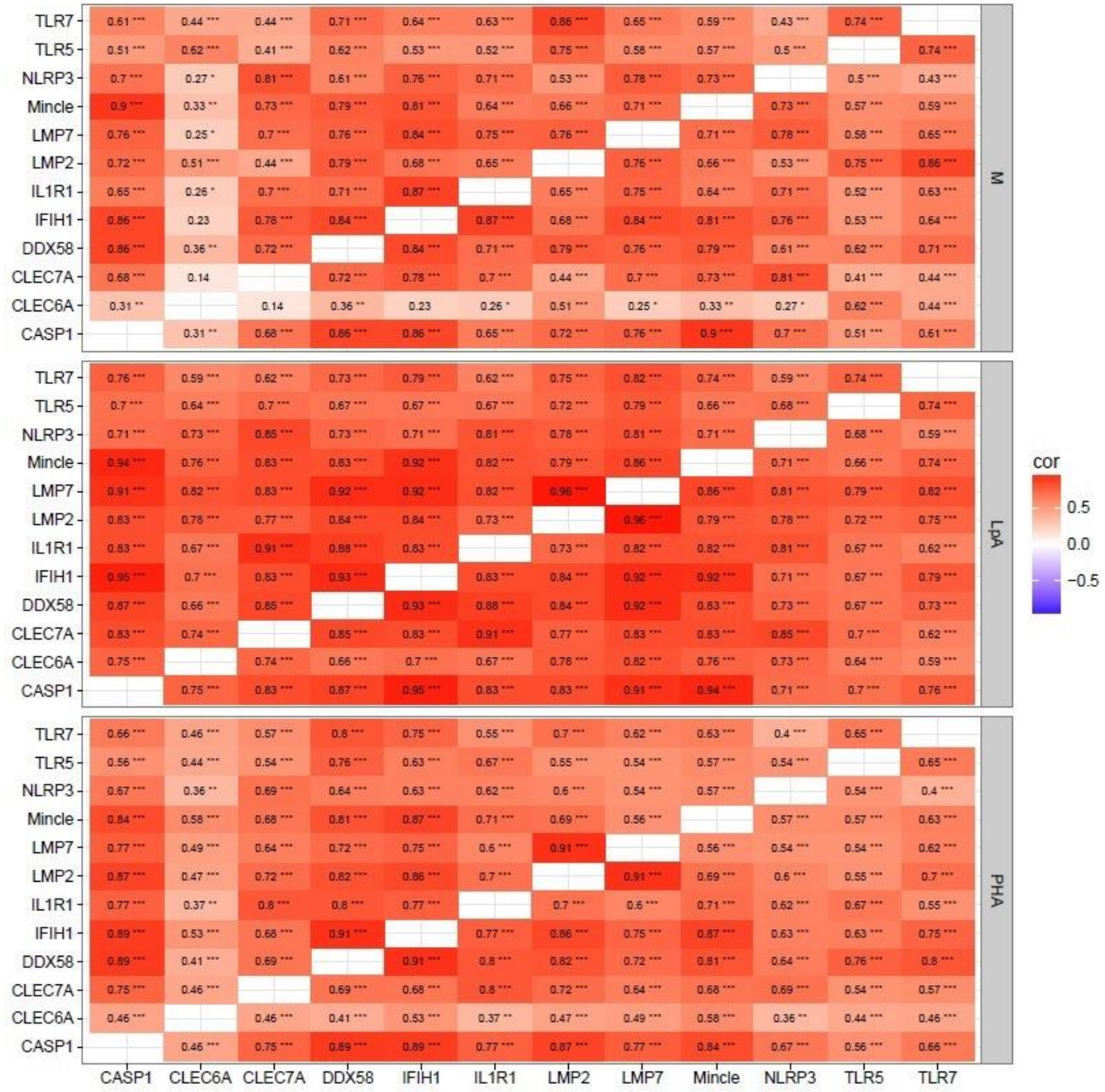


Figure 15: gene-gene correlations

4.3.4. Increased gene expression after CBMC stimulation

The following results show the differences between the stimulation conditions (media meaning unstimulated, PHA and LpA) for the expression of each gene independent of the different phenotype classification.
Wilcoxon signed rank test was calculated based on the null hypothesis that the distribution of x-y (LpA – M; PHA-M) is symmetric around 0.
Boxplots show first and third quartiles (box) and median (line). Whiskers are extended to the most extreme data point that is no more than 1.5 x IQR (inter quartile range) from the edge of the box. Data beyond the end of the whiskers are plotted as points (<1.5 IQR) or stars (< 3 IQR).

4.3.4.1. TLR5

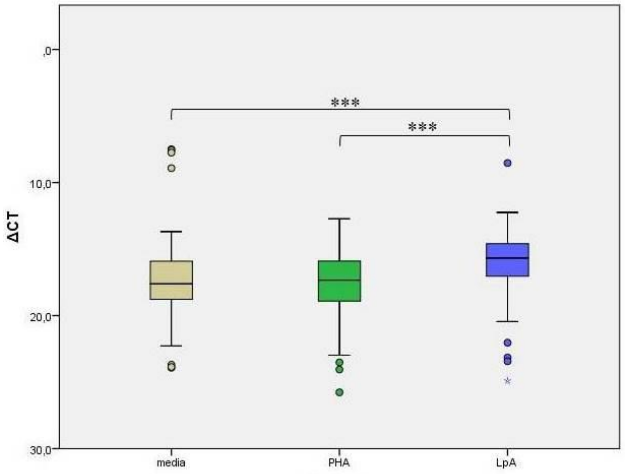


Figure 16: ΔCT of TLR5 in the different stimulation conditions
p-value: ≤0.05=* ≤0.01= ** ≤ 0.005=***

Table 12: median and 95% CI for ΔCT values of TLR5.

TLR5	*n*	*median*	*95% CI*
media	75	17.61	16.55;18.00
PHA	70	17.34	17.01;18.25
LpA	70	15.67	15.49;16.79

TLR5	*p-value*
m vs PHA	0.95
m vs LPA	< 0.001
LpA vs PHA	< 0.001

For TLR5, stimulation with LpA resulted in significantly upregulated gene expression (vs unstimulated and PHA-stimulated cells).

4.3.4.2. TLR7

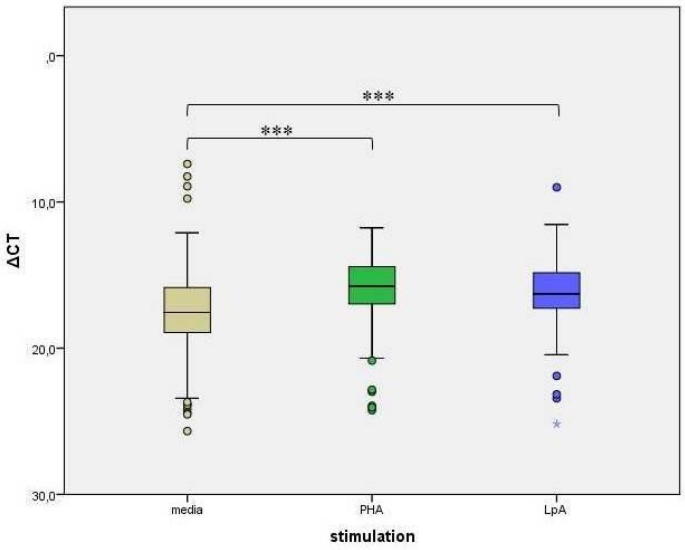


Figure 17: ΔCT of TLR7 in the different stimulation conditions
p-value: ≤0.05=* ≤0.01= ** ≤ 0.005=***

Table 13: median and 95% CI for ΔCT values of TLR7.

TLR7	*n*	*median*	*95% CI*
media	75	17.56	16.82;18.49
PHA	74	15.77	15.66;16.97
LpA	70	16.29	15.78;17.02

TLR7	*p-value*
m vs PHA	0.001
m vs LPA	< 0.001
LpA vs PHA	0.34

Both LpA and PHA stimulation conditions resulted in significant upregulation of TLR7 gene expression.

4.3.4.3. RIG-I (DDX58)

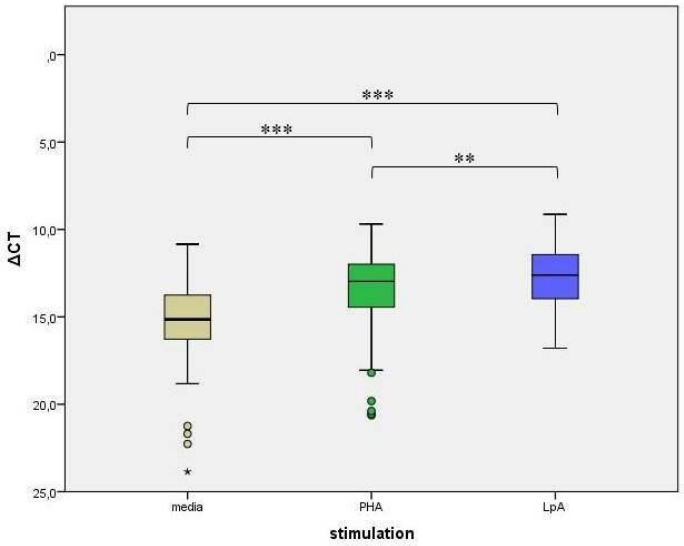


Figure 18: ΔCT of RIG-I in the different stimulation conditions
p-value: ≤0.05=* ≤0.01= ** ≤ 0.005=***

Table 14: median and 95% CI for ΔCT values of RIG-I

RIG-I	*n*	*median*	*95% CI*
media	76	15.15	14.70;15.83
PHA	74	12.97	12.86;14.03
LpA	71	12.61	12.33;13.13

RIG-I	*p-value*
m vs PHA	< 0.001
m vs LPA	< 0.001
LpA vs PHA	0.009

For RIG-I, both stimulation conditions led to a significant upregulation of gene expression, with LpA stimulation resulting in a significantly higher gene expression than PHA.

4.3.4.4. MDA-5 (IFIH1)

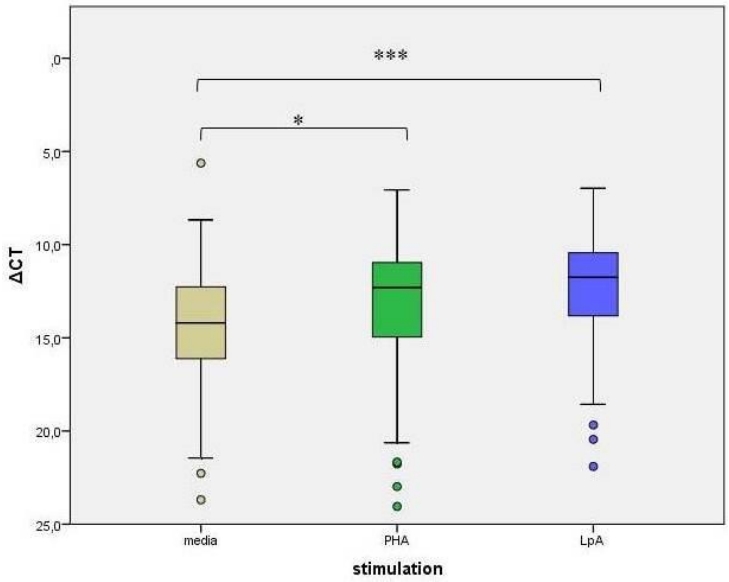


Figure 19: ΔCT of MDA-5 in the different stimulation conditions
p-value: ≤0.05=* ≤0.01= ** ≤ 0.005=***

Table 15: median and 95% CI for ΔCT values of MDA-5

MDA-5	*n*	*median*	*95% CI*
media	75	14.20	13.57;15.08
PHA	75	12.30	12.33;14.04
LpA	72	11.75	11.81;13.21

MDA-5	*p-value*
m vs PHA	0.024
m vs LPA	< 0.001
LpA vs PHA	0.11

For MDA-5, the stimulation with both PHA and LPA resulted in significant upregulation of the gene expression.

4.3.4.5. Mincle (CLEC4E)

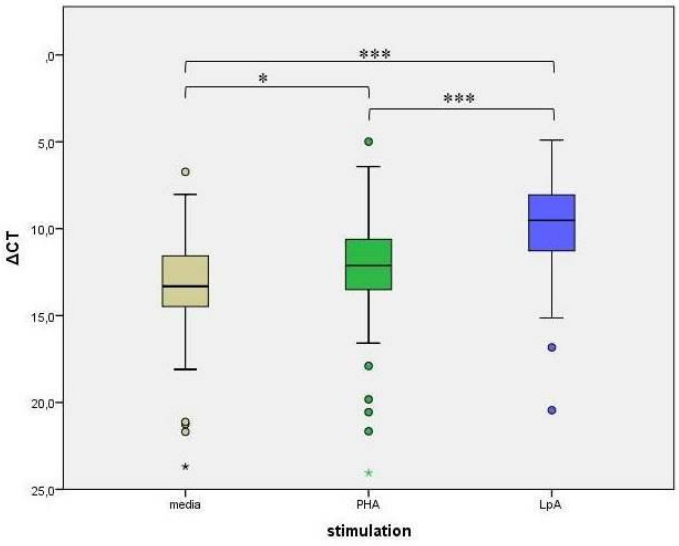


Figure 20: ΔCT of Mincle in the different stimulation conditions
p-value: ≤0.05=* ≤0.01= ** ≤ 0.005=***

Table 16: median and 95% CI for ΔCT values of Mincle.

Mincle	*n*	*median*	*95% CI*
media	74	13.32	12.63;14.04
PHA	72	12.14	11.53;13.07
LpA	72	9.52	9.15;10.43

Mincle	*p-value*
m vs PHA	0.038
m vs LPA	< 0.001
LpA vs PHA	< 0.001

For Mincle, both stimulation conditions resulted in an upregulation of the gene expression with LpA showing the strongest effect.

4.3.4.6. Dectin1 (CLEC7A)

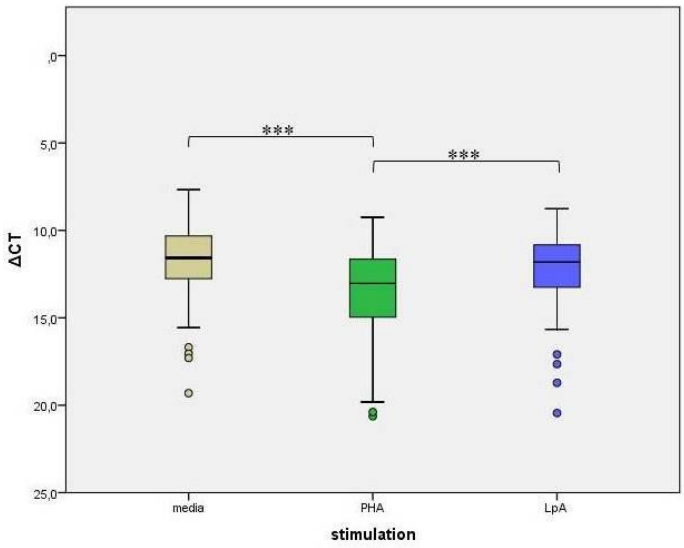


Figure 21: ΔCT of Dectin1 in the different stimulation conditions
p-value: ≤0.05=* ≤0.01= ** ≤ 0.005=***

Table 17: median and 95% CI for ΔCT values of Dectin1.

Dectin1	*n*	*median*	*95% CI*
media	74	11.58	11.33;12.37
PHA	74	13.03	12.97;14.22
LpA	72	11.82	11.68;12.73

Dectin1	*p-value*
m vs PHA	< 0.001
m vs LPA	0.24
LpA vs PHA	< 0.001

For Dectin1, the stimulation with PHA led to a significant downregulation of the gene expression compared with both unstimulated cells and LpA stimulation.

4.3.4.7. Dectin2 (CLEC6A)

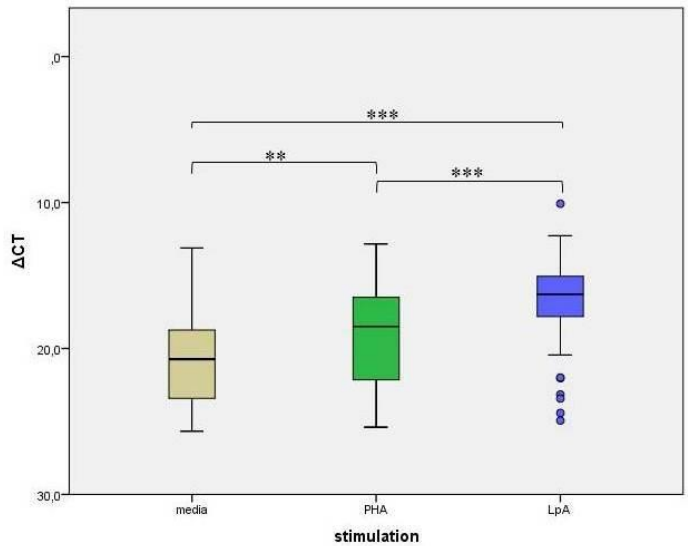


Figure 22: ΔCT of Dectin2 in the different stimulation conditions
p-value: ≤0.05=* ≤0.01= ** ≤ 0.005=***

Table 18: median and 95% CI for ΔCT values of Dectin2.

Dectin2	*n*	*median*	*95% CI*
media	71	20.73	19.99;21.42
PHA	71	18.49	18.36;19.93
LpA	70	16.29	16.15;17.45

Dectin2	*p-value*
m vs PHA	0.007
m vs LPA	< 0.001
LpA vs PHA	< 0.001

For Dectin2, both stimulation conditions affected the gene expression, with LpA stimulation resulting in a significantly higher gene expression than PHA. These findings must be seen in context with the high non-detection rate meaning a limited informative value.

4.3.4.8. LMP2

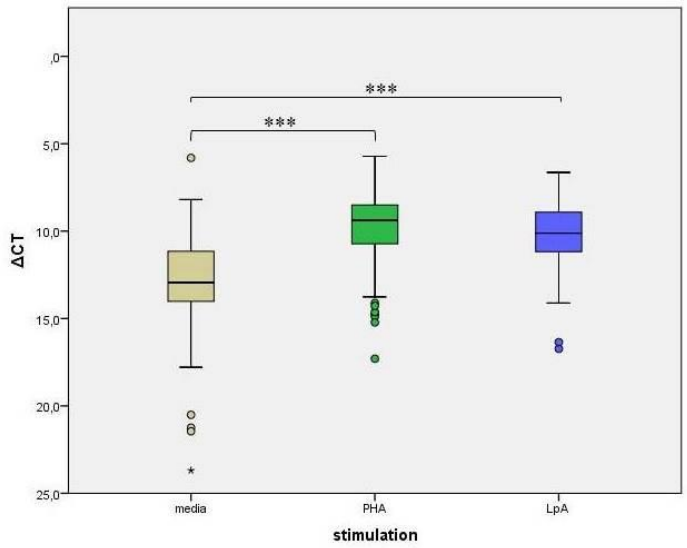


Figure 23: ΔCT of LMP2 in the different stimulation conditions
p-value: ≤0.05=* ≤0.01= ** ≤ 0.005=***

Table 19: median and 95% CI for ΔCT values of LMP2.

LMP2	*n*	*median*	*95% CI*
media	76	12.95	12.03;13.67
PHA	75	9.38	9.44;10.49
LpA	72	10.12	9.76;10.70

LMP2	*p-value*
m vs PHA	< 0.001
m vs LPA	< 0.001
LpA vs PHA	0.29

For LMP2, both stimulation conditions resulted in a significant upregulation of gene expression.

4.3.4.9. LMP7

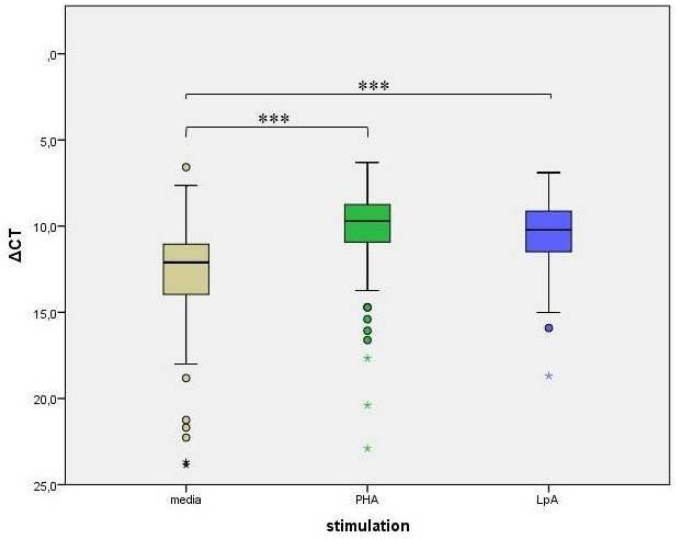


Figure 24: ΔCT of LMP7 in the different stimulation conditions
p-value: ≤0.05=* ≤0.01= ** ≤ 0.005=***

Table 20: median and 95% CI for ΔCT values of LMP7.

LMP7	*n*	*median*	*95% CI*
media	76	12.11	12.26;13.78
PHA	73	9.71	9.77;11.17
LpA	69	10.22	10.05;11.09

LMP7	*p-value*
m vs PHA	< 0.001
m vs LPA	< 0.001
LpA vs PHA	0.17

For LMP7, both stimulation conditions resulted in a significant upregulating effect on the gene expression.

4.3.4.10. NLRP3

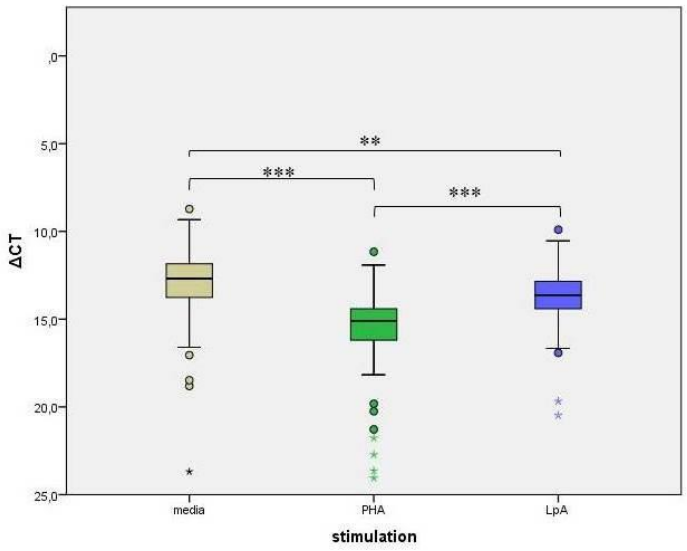


Figure 25: ΔCT of NLRP3 in the different stimulation conditions
p-value: ≤0.05=* ≤0.01= ** ≤ 0.005=***

Table 21: median and 95% CI for ΔCT values of NLRP3.

NLRP3	*n*	*median*	*95% CI*
media	76	12.69	12.52;13.53
PHA	73	15.10	15.17;16.31
LpA	72	13.64	13.29;14.10

NLRP3	*p-value*
m vs PHA	< 0.001
m vs LPA	0.0058
LpA vs PHA	< 0.001

For NLRP3, both stimulation conditions led to a significant downregulation of gene expression with PHA showing the strongest effect.

4.3.4.11. Casp1

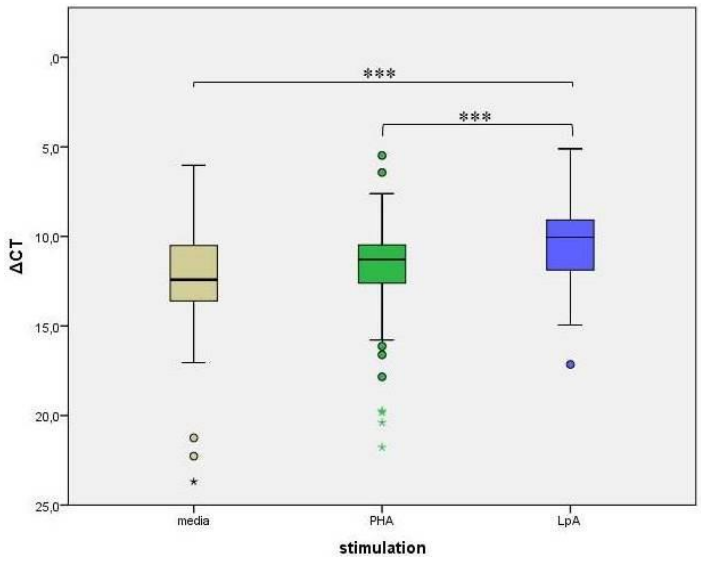


Figure 26: ΔCT of Casp1 in the different stimulation conditions
p-value: ≤0.05=* ≤0.01= ** ≤ 0.005=***

Table 22: median and 95% CI for ΔCT values of Casp1.

Casp1	*n*	*median*	*95% CI*
media	75	12.42	11.76;13.17
PHA	75	11.30	11.10;12.48
LpA	72	10.05	9.87;11.00

Casp1	*p-value*
m vs PHA	0.16
m vs LPA	< 0.001
LpA vs PHA	< 0.001

For Casp1, the stimulation with LPA resulted in significant upregulation of the gene expression.

4.3.4.12. IL-1R1

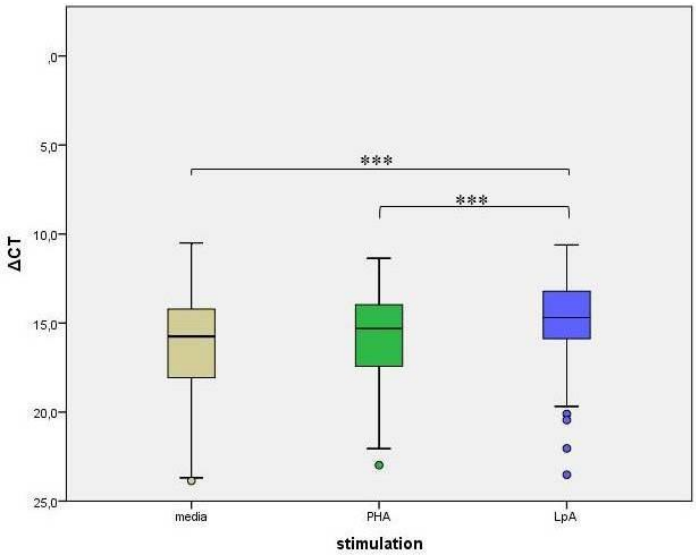


Figure 27: ΔCT of IL-1R1 in the different stimulation conditions
p-value: ≤0.05=* ≤0.01= ** ≤ 0.005=***

Table 23: median and 95% CI for ΔCT values of IL-1R1.

IL-1R1	*n*	*median*	*95% CI*
media	75	15.75	15.43;16.81
PHA	75	15.31	15.26;16.45
LpA	71	14.69	14.30;15.45

IL-1R1	*p-value*
m vs PHA	0.88
m vs LPA	< 0.001
LpA vs PHA	< 0.001

For IL-1R1, the stimulation with LPA resulted in significant upregulation of the gene expression.

4.3.5. Differences in gene expression of the innate immune system among the wheeze phenotypes

The following results show the difference between the gene expression of the phenotypes defined according to 4.1.
The following abbreviations were used:
HC=healthy control (n=26) EVW= early viral wheeze (n=18)
MT=multitrigger wheeze (n=14) VW= viral wheeze (persistent and late onset) (n=18)
To test for difference of the gene expression between the different wheeze phenotypes, Mann-Whitney-U test was performed.
Boxplots show first and third quartiles (box) and median (line). Whiskers are extended to the most extreme data point that is no more than 1.5 x IQR (inter quartile range) from the edge of the box. Data beyond the end of the whiskers are plotted as points (<1.5 IQR) or stars (< 3 IQR).

4.3.5.1. TLR5

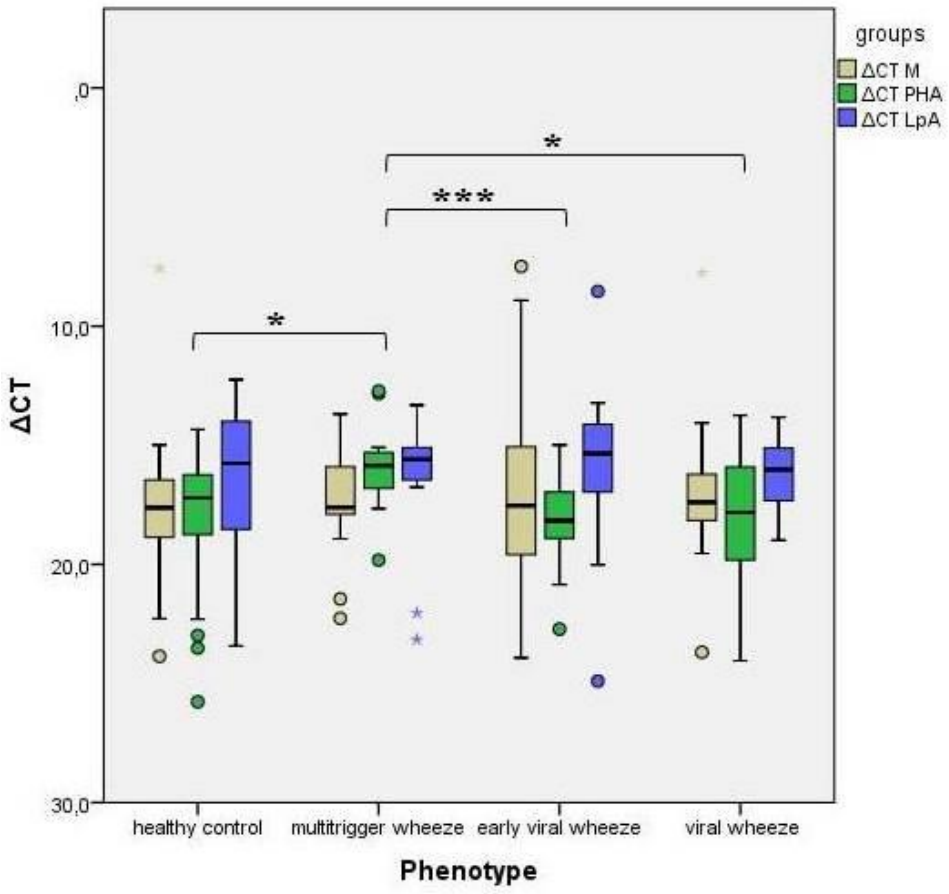


Figure 28: Difference between the wheeze phenotypes for the gene expression of TLR5.
p-value: ≤0.1= # ≤ 0.05= * ; ≤ 0.01=** ,≤ 0.005=***

For TLR5, there was a significant difference detectable following PHA stimulation (in green). **MT** wheeze showed the highest gene expression compared **with HC (p-value= 0.020), EVW (p-value=0.0028) and VW (p-value= 0.028)**.

Table 24: Difference between the wheeze phenotypes for the gene expression of TLR5 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

TLR5		*n*	*median*	*95% CI*
HC	*M*	26	17.63	16.35;18.68
	PHA	24	17.21	16.80;19.33
	LpA	26	15.77	15.06;17.23
MT	*M*	13	17.61	15.98;19.00
	PHA	13	15.86	14.77;17.03
	LPA	12	15.59	14.57;18.41
EVW	*M*	18	17.54	15.03;19.12
	PHA	17	18.17	17.11;19.05
	LPA	18	15.35	14.15;17.54
VW	*M*	18	17.40	15.45;18.53
	PHA	16	17.83	16.45;19.37
	LPA	14	16.02	15.29;17.13

4.3.5.2. TLR7

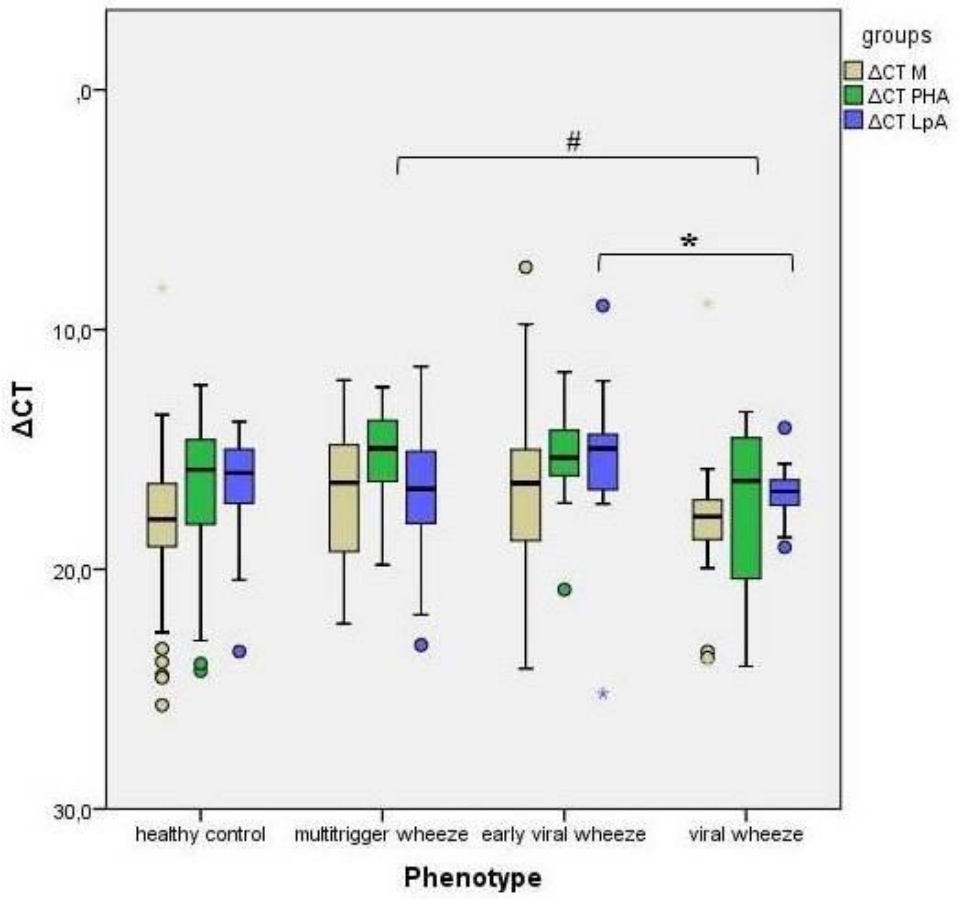


Figure 29: Difference between the wheeze phenotypes for the gene expression of TLR7.
p-value: ≤0.1= # ≤0.05= * ; ≤0.01=** ,≤0.005=***

For TLR7, there was a significant upregulation for **EVW** compared to **VW** after LpA stimulation **(p-value= 0.012)** detectable. Furthermore, there was a trend towards higher gene expression in MT compared with VW following PHA-stimulation (p-value=0.092).

Table 25: Difference between the wheeze phenotypes for the gene expression of TLR7 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

TLR7		*n*	*median*	*95% CI*
HC	*M*	25	17.92	16.80;20.02
	PHA	26	15.85	15.69;18.20
	LpA	26	15.98	15.66;17.52
MT	*M*	14	16.41	15.25;18.66
	PHA	14	14.96	14.16;16.29
	LPA	11	16.65	14.76;19.26
EVW	*M*	18	16.40	14.69;18.55
	PHA	17	15.35	14.29;16.37
	LPA	18	14.97	13.91;17.02
VW	*M*	18	17.81	16.50;19.86
	PHA	17	16.31	15.56;18.95
	LPA	15	16.75	16.12;17.45

4.3.5.3. RIG-I (DDX58)

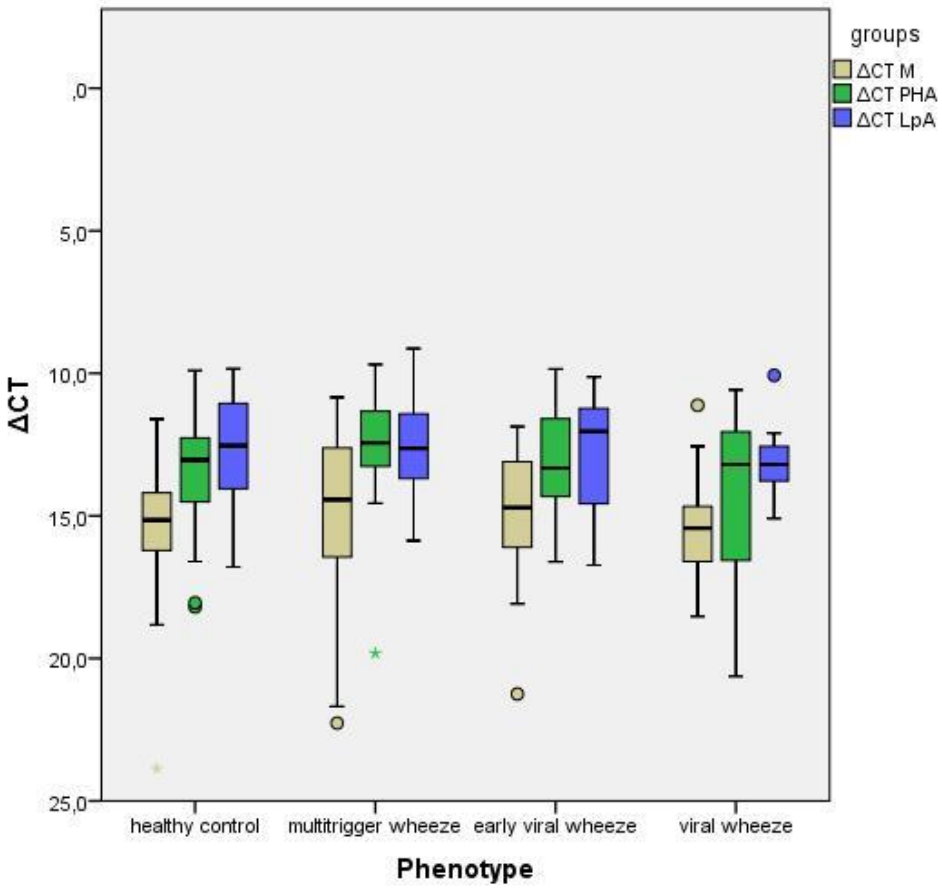


Figure 30: Difference between the wheeze phenotypes for the gene expression of RIG-I.
p-value: ≤0.1= # ≤0.05= * ; ≤0.01=** ,≤0.005=***

For RIG-I, there were no statistically significant differences between the phenotypes detectable.

Table 26: Difference between the wheeze phenotypes for the gene expression of RIG-I on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

RIG-I		*n*	*median*	*95% CI*
HC	*M*	26	15.15	14.45;16.33
	PHA	26	13.04	12.61;14.38
	LpA	26	12.54	11.89;13.30
MT	*M*	14	14.43	13.28;17.23
	PHA	14	12.44	11.31;14.13
	LPA	11	12.63	11.33;13.80
EVW	*M*	18	14.71	13.57;15.97
	PHA	18	13.33	12.03:13.93
	LPA	18	12.04	11.68;13.68
VW	*M*	18	15.44	14.62;16.56
	PHA	16	13.20	12.68;16.33
	LPA	16	13.20	12.49;13.71

4.3.5.4. MDA-5 (IFIH1)

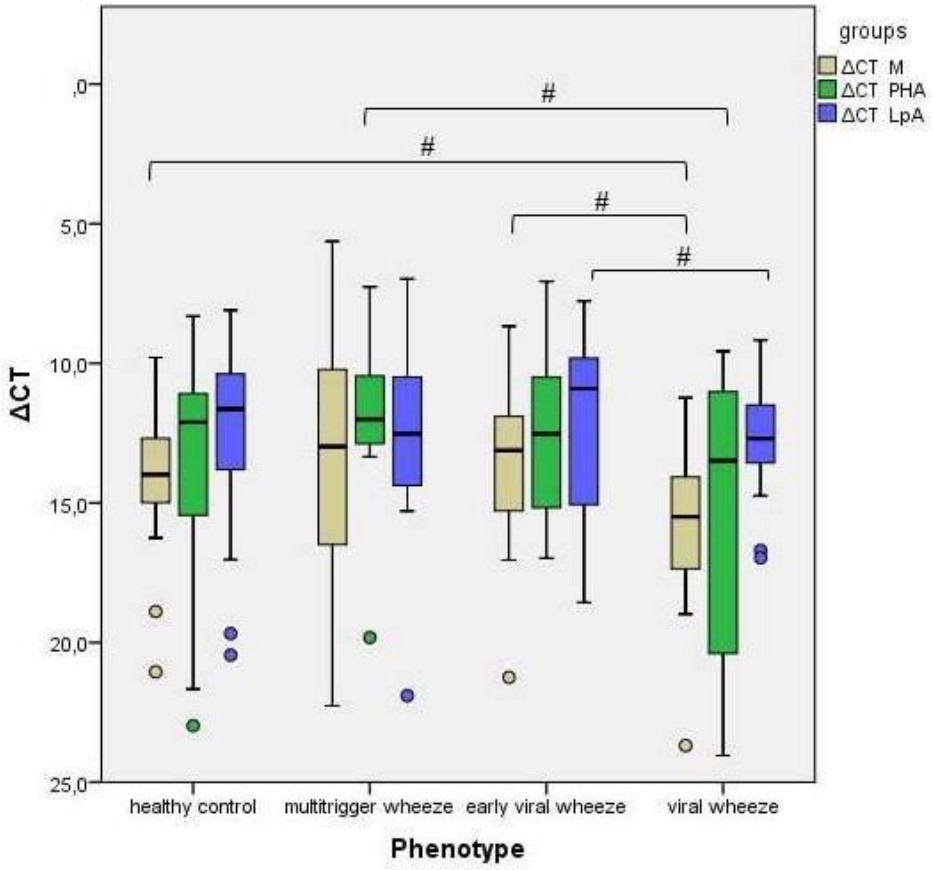


Figure 31: Difference between the wheeze phenotypes for the gene expression of MDA-5.
p-value : ≤0.1= # ≤0.05= * ; ≤0.01=** ,≤0.005=***

For MDA-5 there was a trend for a lower gene expression in VW in media compared with EVW (p-value=0.066) and HC (p-value=0.052), after PHA stimulation compared with

MT (p-value= 0.062) and following LpA stimulation compared with EVW (p-value= 0.081), although not statistically significant.

Table 27: Difference between the wheeze phenotypes for the gene expression of MDA-5 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

MDA-5		*n*	*median*	*95% CI*
HC	*M*	26	13.99	13.04;15.04
	PHA	26	12.11	11.90;14.80
	LpA	26	11.64	11.25;13.72
MT	*M*	14	12.98	11.19;16.57
	PHA	14	12.01	10.10;13.52
	LPA	12	12.53	10.44;15.14
EVW	*M*	17	13.12	12.08;15.32
	PHA	18	12.52	10.94;13.83
	LPA	18	10.91	10.42;13.63
VW	*M*	18	15.50	14.18;17.20
	PHA	17	13.49	12.47;17.33
	LPA	16	12.70	11.80;13.93

4.3.5.5. Mincle (CLEC4E)

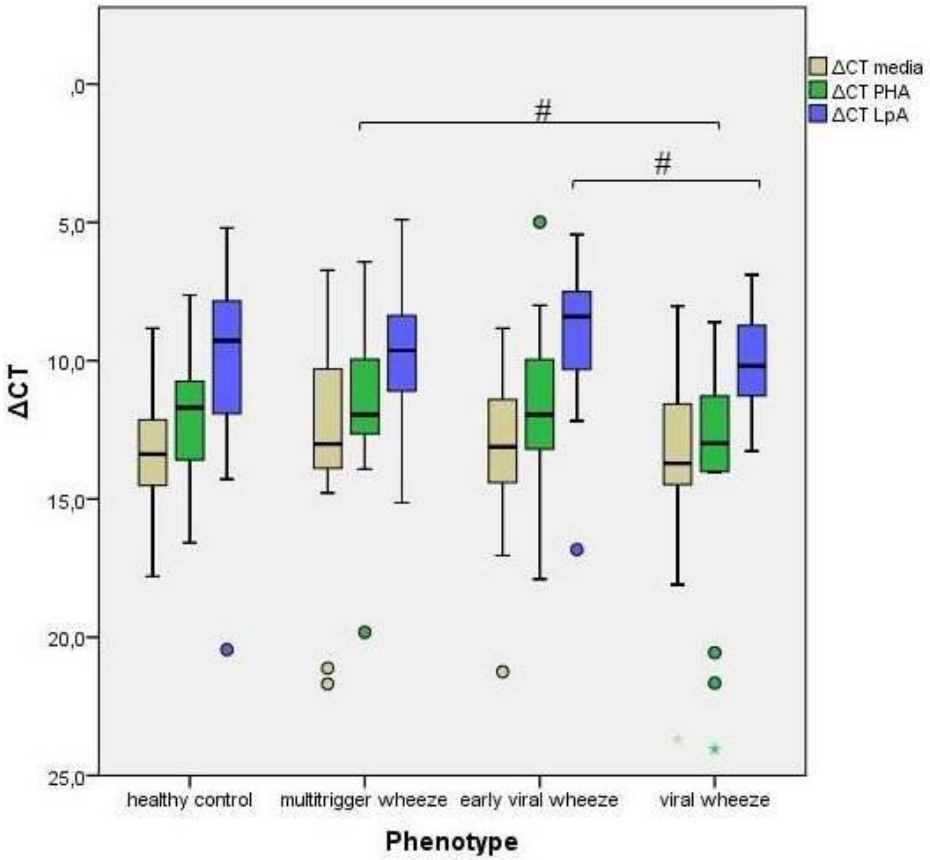


Figure 32: Difference between the wheeze phenotypes for the gene expression of Mincle.
p-value : ≤0.1= # ≤ 0.05= * ; ≤ 0.01=** ,≤ 0.005=***

For Mincle, there was a trend for a lower gene expression in VW after PHA stimulation compared with MT (p-value=0.081) and after LpA stimulation compared with EVW (p-value= 0.055), although not statistically significant.

Table 28: Difference between the wheeze phenotypes for the gene expression of Mincle on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

Mincle		*n*	*mean*	*95% CI*
HC	*M*	25	13.38	12.59;14.16
	PHA	25	11.96	11.11;13.08
	LpA	26	9.28	8.68;11.32
MT	*M*	14	13.01	1.61;15.44
	PHA	14	11.95	9.65;13.42
	LPA	12	9.64	8.20;11.47
EVW	*M*	17	13.12	11.60;14.65
	PHA	18	11.95	10.24;13.15
	LPA	18	8.41	7.71;10.43
VW	*M*	18	13.72	11.96;15.48
	PHA	15	12.99	11.61;16.54
	LPA	16	10.19	9.25;11.18

4.3.5.6. Dectin1 (CLEC7A)

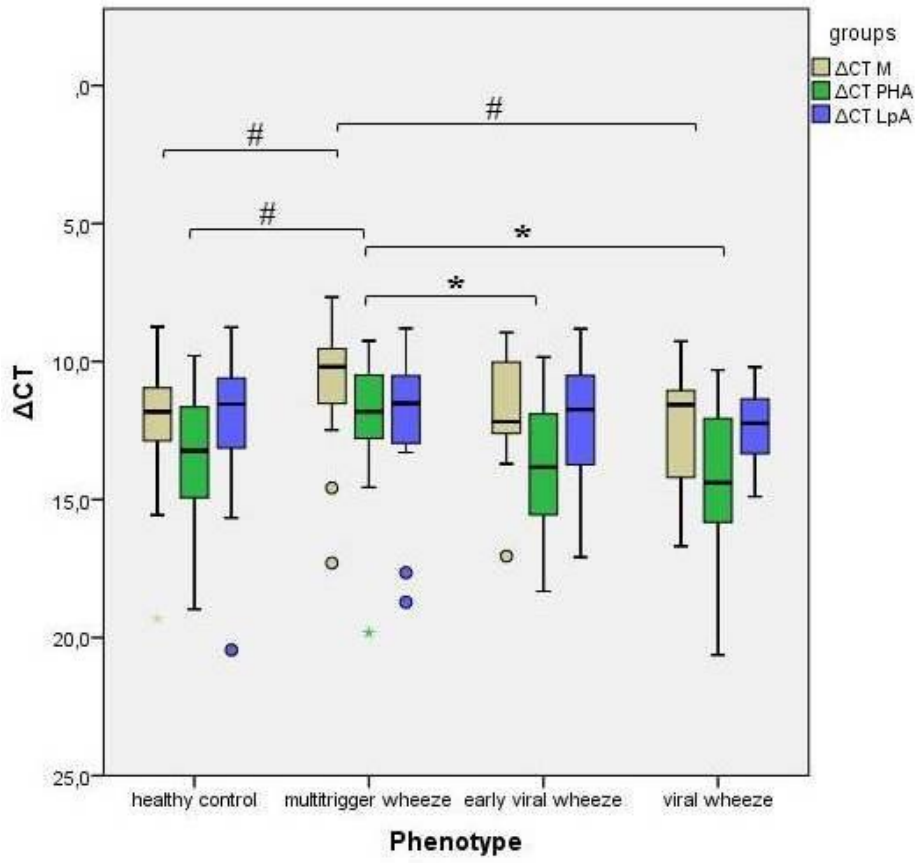


Figure 33: Difference between the wheeze phenotypes for the gene expression of Dectin1
p-value : ≤0.1= # ≤0.05= * ; ≤0.01=** ,≤0.005=***

For Dectin1, there was a significantly higher gene expression in **MT** following PHA stimulation compared **with VW (p-value= 0.012) and with EVW (p-value= 0.037)** and a trend for higher gene expression compared with HC (p-value=0.063). The gene expression in MT also tended to be higher in media compared with VW (p-value=0.097) and with HC (p-value=0.051).

Table 29: Difference between the wheeze phenotypes for the gene expression of Dectin1 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

Dectin1		*n*	*median*	*95% CI*
HC	*M*	26	11.82	11.28;13.08
	PHA	25	13.23	12.55;14.50
	LpA	26	11.54	11.07;13.05
MT	*M*	13	10.19	9.29;12.55
	PHA	14	11.82	10.62;13.71
	LPA	12	11.52	10.46;14.24
EVW	*M*	17	12.12	10.67;12.78
	PHA	18	13.83	12.56;14.89
	LPA	18	11.75	11.09;13.23
VW	*M*	18	11.57	11.15;13.18
	PHA	17	14.39	13.10;16.40
	LPA	16	12.24	11.71;13.06

4.3.5.7. Dectin2 (CLEC6A)

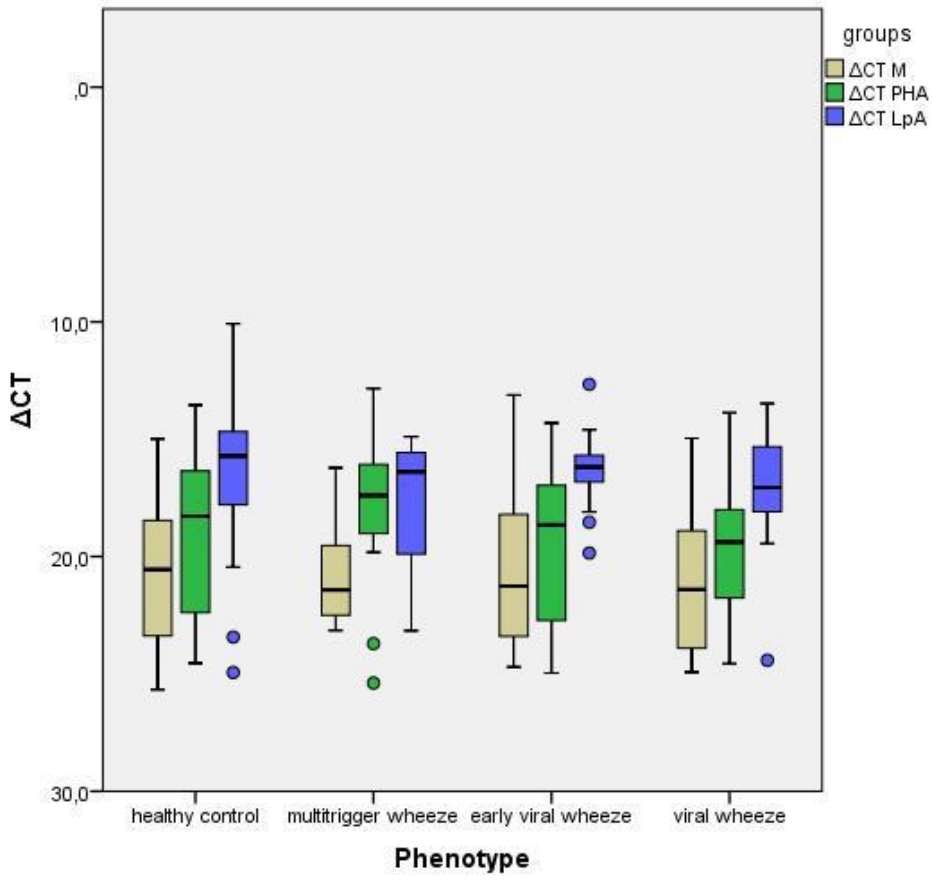


Figure 34: Difference between the wheeze phenotypes for the gene expression of Dectin2.
p-value: ≤0.1= # ≤0.05= * ; ≤0.01=** ,≤0.005=***

For Dectin2, there were no statistically significant differences identifiable between the wheeze phenotypes.

Table 30: Difference between the wheeze phenotypes for the gene expression of Dectin2 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

Dectin2		*n*	*median*	*95% CI*
HC	*M*	24	20.55	19.27;21.85
	PHA	24	18.28	17.59;20.52
	LpA	25	15.71	15.15;17.84
MT	*M*	11	21.42	19.20;22.19
	PHA	12	17.40	15.85;20.29
	LPA	12	16.40	15.83;19.60
EVW	*M*	18	21.26	18.94;22.19
	PHA	18	18.65	17.85;21.19
	LPA	17	16.19	15.54;17.23
VW	*M*	18	21.40	19.37;22.70
	PHA	17	19.38	18.06;21.21
	LPA	16	17.06	15.65;18.43

4.3.5.8. LMP2

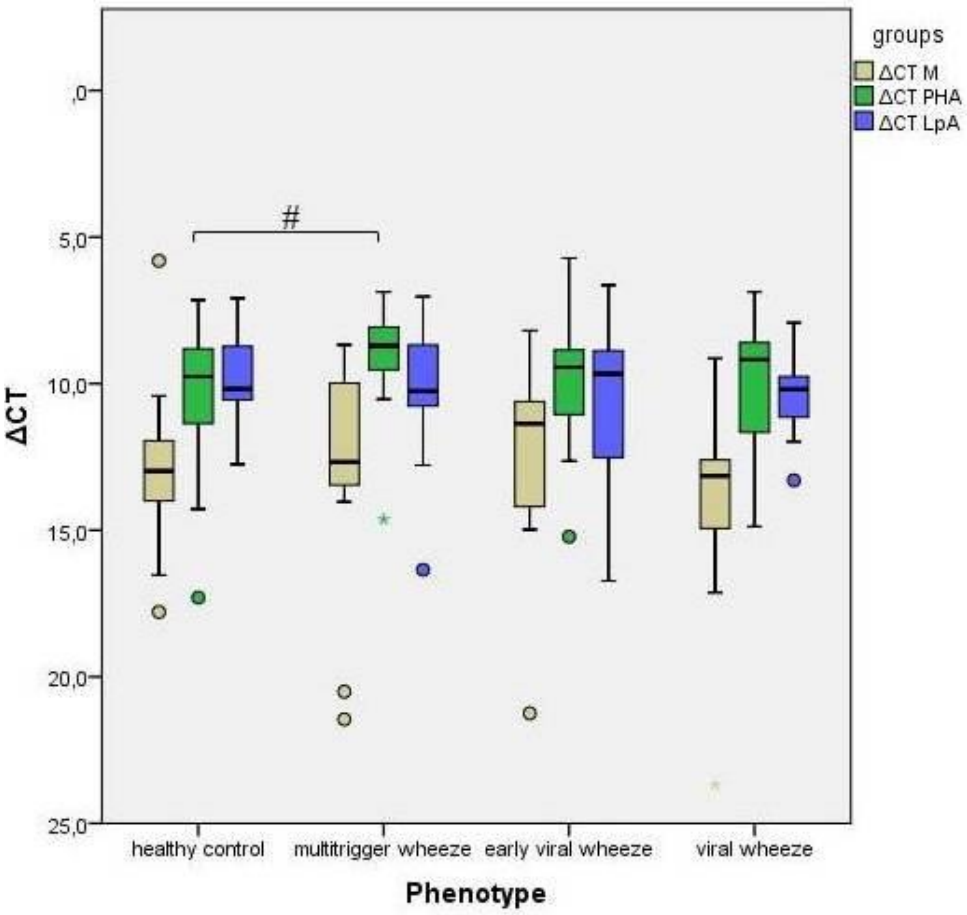


Figure 35: Difference between the wheeze phenotypes for the gene expression of LMP2.
p-value: ≤0.1= # ≤ 0.05= * ; ≤ 0.01=** ,≤ 0.005=***

For LMP2, there was a trend for a higher gene expression in MT compared with HC (p-value=0.056) after PHA stimulation, although not statistically significant.

Table 31: Difference between the wheeze phenotypes for the gene expression of LMP2 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

LMP2		*n*	*median*	*95% CI*
HC	*M*	26	12.98	12.08;13.90
	PHA	26	9.76	9.36;11.24
	LpA	26	10.19	9.25;10.64
MT	*M*	14	12.68	10.54;15.07
	PHA	14	8.71	8.01;10.24
	LPA	12	10.26	8.72;11.86
EVW	*M*	18	11.37	10.92;13.83
	PHA	18	9.45	8.82;10.94
	LPA	18	9.67	9.13;11.73
VW	*M*	18	13.15	12.11;15.35
	PHA	17	9.18	8.91;11.59
	LPA	16	10.19	9.76;11.10

4.3.5.9. LMP7

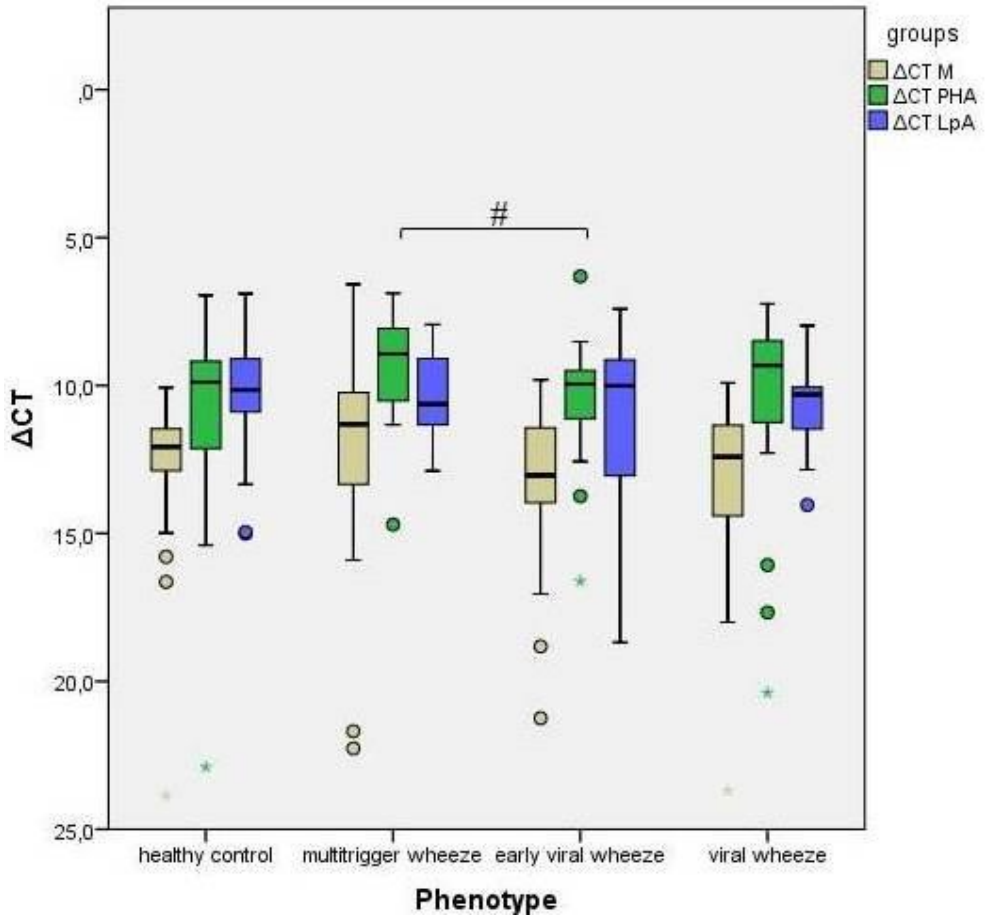


Figure 36: Difference between the wheeze phenotypes for the gene expression of LMP7.
p-value: ≤0.1= # ≤0.05= * ; ≤0.01=** ,≤0.005=***

For LMP7, there was a trend for a higher gene expression in MT compared with EVW (p-value=0.084) following PHA stimulation, although not statistically significant.

Table 32: Difference between the wheeze phenotypes for the gene expression of LMP7 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

LMP7		*n*	*median*	*95% CI*
HC	*M*	26	12.08	11.58;13.87
	PHA	26	9.89	9.51;12.13
	LpA	25	10.15	9.43;11.12
MT	*M*	14	11.32	9.81;15.17
	PHA	14	8.94	8.17;10.52
	LPA	11	10.63	9.19;11.42
EVW	*M*	18	13.04	11.81;14.84
	PHA	17	9.95	9.39;11.76
	LPA	17	10.01	9.38;12.56
VW	*M*	18	12.41	11.90;15.18
	PHA	16	9.33	8.71;12.84
	LPA	16	10.31	10.02;11.57

4.3.5.10. NLRP3

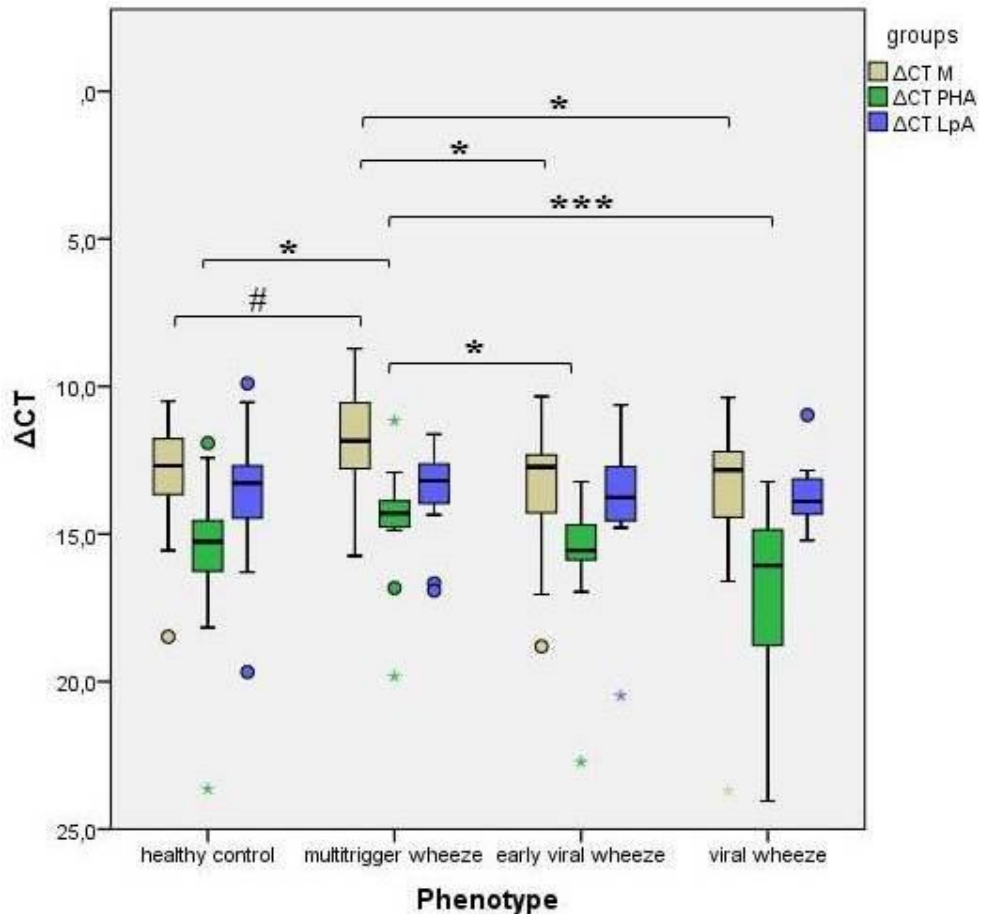


Figure 37: Difference between the wheeze phenotypes for the gene expression of NLRP3.
p-value: ≤0.1= # ≤0.05= * ; ≤0.01=** ,≤0.005=***

For NLRP3 there was a significant higher gene expression in **MT** in both media compared **with VW (p-value=0.028), EVW (p-value=0.024)** a trend compared with HC (p-value=0.081) and after PHA stimulation in **MT** compared **with VW (p-value=0.005), EVW (p-value=0.013) and HC (p -value=0.015).**

Table 33: Difference between the wheeze phenotypes for the gene expression of NLRP3 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

NLRP3		*n*	*median*	*95% CI*
HC	*M*	26	12.69	12.21;13.57
	PHA	26	15.26	14.74;16.54
	LpA	26	13.28	12.78;14.38
MT	*M*	14	11.85	10.71;12.88
	PHA	14	14.30	13.39;15.65
	LPA	12	13.20	12.62;14.68
EVW	*M*	18	12.73	12.43;14.53
	PHA	17	15.56	14.65;16.74
	LPA	18	13.77	12.87;14.83
VW	*M*	18	12.84	12.28;15.18
	PHA	16	16.07	15.34;18.67
	LPA	16	13.90	13.19;14.30

4.3.5.11. Casp1

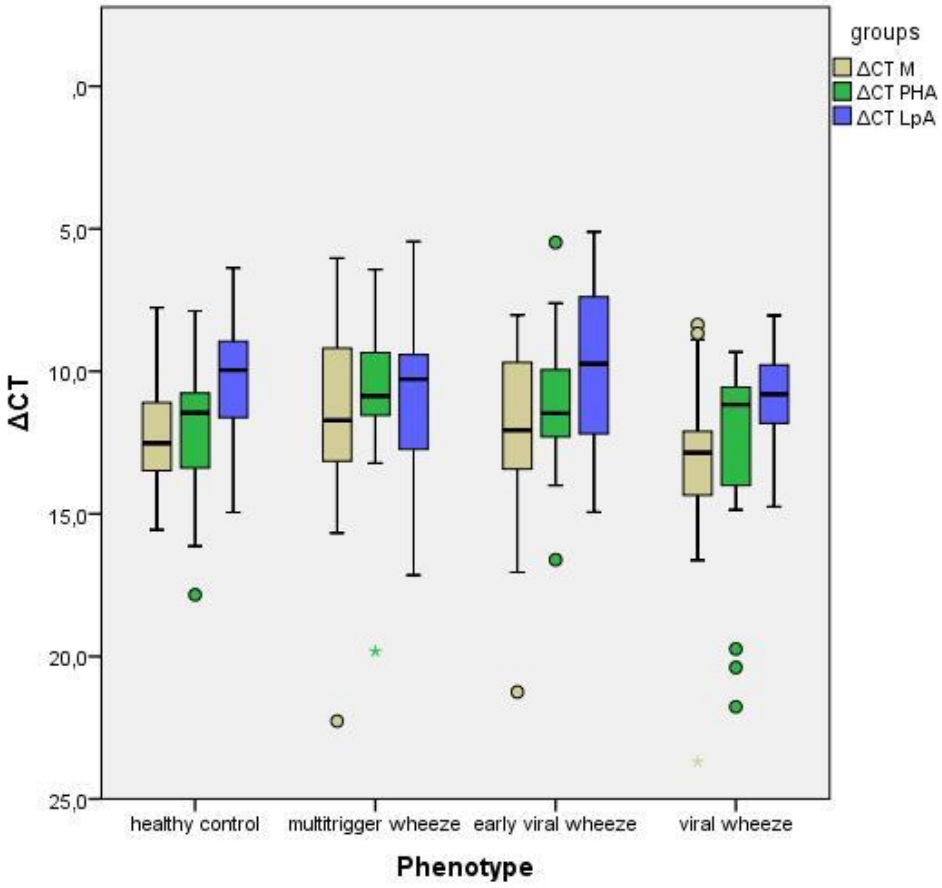


Figure 38: Difference between the wheeze phenotypes for the gene expression of Casp1.
p-value: ≤0.1= # ≤ 0.05= * ; ≤ 0.01=** ,≤ 0.005=***

For Casp1, there were no statistically significant differences identifiable between the wheeze phenotypes.

Table 34: Difference between the wheeze phenotypes for the gene expression of Casp1 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

Casp1		*n*	*median*	*95% CI*
HC	*M*	25	12.52	11.67;13.20
	PHA	26	11.46	10.95;12.85
	LpA	26	9.96	9.40;11.20
MT	*M*	14	11.71	9.60;14.13
	PHA	14	10.87	9.23;12.79
	LPA	12	10.28	8.98;12.80
EVW	*M*	18	12.07	10.56;13.87
	PHA	18	11.48	9.82;12.37
	LPA	18	9.74	8.48;11.13
VW	*M*	18	12.86	11.51;14.97
	PHA	17	11.17	10.98;15.04
	LPA	16	10.98	10.08;11.95

4.3.5.12. IL-1R1

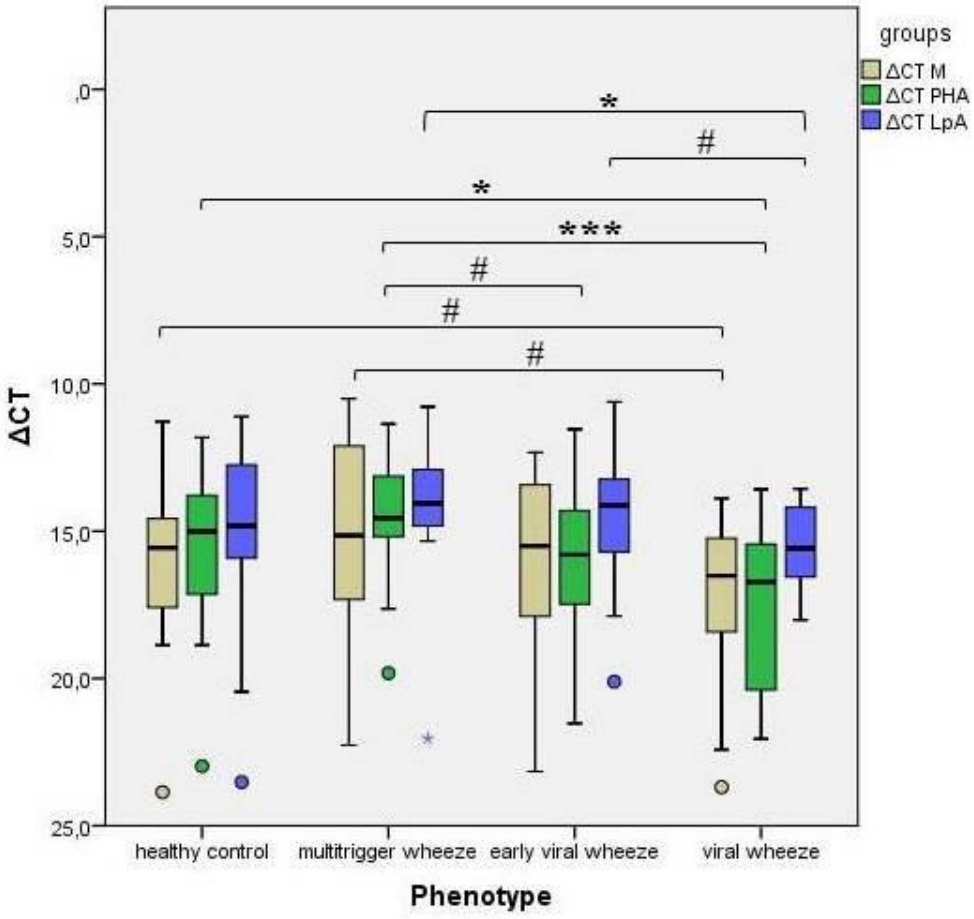


Figure 39: Difference between the wheeze phenotypes for the gene expression of IL-1R1.
p-value: ≤0.1= # ≤ 0.05= * ; ≤ 0.01=** ,≤ 0.005=***

For IL-1R1 there was a trend for a lower expression in VW in media compared with MT (p-value=0.071) and with HC (p-value=0.055). After PHA stimulation, the gene expression in **MT** was significantly higher compared **with VW (p-value=0.003)** and tended to be higher compared with EVW (p-value=0.08). Additionally, the expression was significantly lower in **VW** compared **with HC (p-value=0.033)**. After LpA stimulation, the gene expression was significantly lower in **VW** compared with **MT (p-value=0.03)** and tended to be lower compared with EVW (p-value=0.076).

Table 35: Difference between the wheeze phenotypes for the gene expression of IL-1R1 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

IL-1R1		*n*	*median*	*95% CI*
HC	*M*	25	15.57	14.77;16.93
	PHA	26	15.02	14.63;16.58
	LpA	26	14.82	13.78;16.13
MT	*M*	14	15.15	13.10;17.54
	PHA	14	14.56	13.27;15.79
	LPA	11	14.06	12.45;16.30
EVW	*M*	18	15.51	14.40;17.48
	PHA	18	15.80	14.67;17.00
	LPA	18	14.13	13.44;15.65
VW	*M*	18	16.52	15.99;18.61
	PHA	17	16.72	15.93;18.79
	LPA	16	15.59	14.78;16.18

4.3.6. Gene expression patterns

Table 36 shows a general overview of gene expression patterns. Significant results are marked in green (p-value <0,05); trends are marked in orange (p-value < 0,1).

Table 36: Overview of gene expression patterns

	TLR5			TLR7			RIG-I			MDA-5		
	media	PHA	LpA	media	PHA	LpA	media	PHA	LpA	media	PHA	LpA
MT vs VW	MT ↓	MT ↑	MT ↑	MT ↑	MT ↑	MT ↑	MT ↑	MT ↑	MT ↑	MT ↑	MT ↑	MT ↑
MT vs EVW	MT ↓	MT ↑	MT ↓	MT ↓	MT ↑	MT ↓	MT ↑	MT ↑	MT ↓	MT ↑	MT ↑	MT ↓
MT vs HC	MT ↑	MT ↑	MT ↑	MT ↑	MT ↑	MT ↓	MT ↑	MT ↑	MT ↓	MT ↑	MT ↑	MT ↓
VW vs EVW	VW ↑	VW↑	VW↓	VW↓	VW↓	VW↓	VW↓	VW↑	VW↓	VW↓	VW↓	VW↓
VW vs HC	VW↑	VW↓	VW↓	VW↑	VW↓	VW↓	VW↓	VW↓	VW↓	VW↓	VW↓	VW↓
EVW vs HC	EVW ↑	EVW ↓	EVW ↑	EVW ↑	EVW ↑	EVW ↑	EVW ↑	EVW ↓	EVW ↑	EVW ↑	EVW ↓	EWV ↑

	Mincle			Dectin1			Dectin2			LMP2		
	media	PHA	LpA	media	PHA	LpA	media	PHA	LpA	media	PHA	LpA
MT vs VW	MT ↑	MT ↑	MT ↑	MT ↑	MT ↑	MT ↑	MT ↓	MT ↑	MT ↑	MT ↑	MT ↑	MT ↓
MT vs EVW	MT ↑	MT ↑	MT ↓	MT ↑	MT ↑	MT ↑	MT ↓	MT ↑	MT ↓	MT ↓	MT ↑	MT ↓
MT vs HC	MT ↑	MT ↓	MT ↓	MT ↑	MT ↑	MT ↑	MT ↓	MT ↑	MT ↓	MT ↑	MT ↑	MT ↓
VW vs EVW	VW↓	VW↓	VW↓	VW↑	VW↓	VW↓	VW↓	VW↓	VW↓	VW↓	VW↑	VW↓
VW vs HC	VW↓	VW↓	VW↓	VW↑	VW↓	VW↓	VW↓	VW↓	VW↓	VW↓	VW↑	VW ↔
EVW vs HC	EVW ↑	EVW ↓	EVW ↑	EVW ↓	EVW ↓	EVW ↓	EVW ↓	EVW ↓	EVW ↓	EVW ↑	EVW ↑	EVW ↑

	LMP7			NLRP3			Casp1			IL-1R1		
	media	PHA	LpA	media	PHA	LpA	media	PHA	LpA	media	PHA	LpA
MT vs VW	MT ↑	MT ↑	MT ↓	MT ↑	MT ↑	MT ↑	MT ↑	MT ↑	MT ↑	MT ↑	MT ↑	MT ↑
MT vs EVW	MT ↑	MT ↑	MT ↓	MT ↑	MT ↑	MT ↑	MT ↑	MT ↑	MT ↓	MT ↑	MT ↑	MT ↑
MT vs HC	MT ↑	MT ↑	MT ↓	MT ↑	MT ↑	MT ↑	MT ↑	MT ↑	MT ↓	MT ↑	MT ↑	MT ↑
VW vs EVW	VW↑	VW↑	VW↓	VW↓	VW↓	VW↓	VW↓	VW↑	VW↓	VW↓	VW↓	VW↓
VW vs HC	VW↓	VW↑	VW↓	VW↓	VW↓	VW↓	VW↓	VW↑	VW↓	VW↓	VW↓	VW↓
EVW vs HC	EVW ↓	EVW ↓	EVW ↑	EVW ↓	EVW ↓	EVW ↓	EVW ↑	EVW ↓	EVW ↑	EVW ↑	EVW ↓	EVW ↑

4.3.7. Differentially expressed genes of the innate immune system considering temporal aspects of wheezing symptoms

In a second step, the phenotypes were divided into more precise groups. Here, the variation over time was factored into analysis.
This resulted in the phenotypes *'persistent multitrigger wheeze'* (multitrigger wheeze at both age 3 and age 6), *'late onset multitrigger wheeze'* (viral wheeze or healthy at age 3, multitrigger wheeze at age 6), *'early viral wheeze'* (viral wheeze at age 3, healthy at age 6), *'persistent viral wheeze'* (viral wheeze at age 3 and age 6) and *'late onset viral wheeze'* (healthy at age 3, viral wheeze at age 6), see 4.1. This led to a smaller sample size within the subgroups (see below).
The following abbreviations were used:

HC=healthy control (n=26)
PMT=persistent multitrigger wheeze (n=4)
LOM=late onset multitrigger wheeze (n=10)
EVW=early viral wheeze (n=18)
PVW=persistent viral wheeze (n=11)
LOVW=late onset viral wheeze (n=6)

Boxplots show first and third quartiles (box) and median (line). Whiskers are extended to the most extreme data point that is no more than 1.5 x IQR (inter quartile range) from the edge of the box. Data beyond the end of the whiskers are plotted as points (<1.5 IQR) or stars (< 3 IQR).

4.3.7.1. TLR5

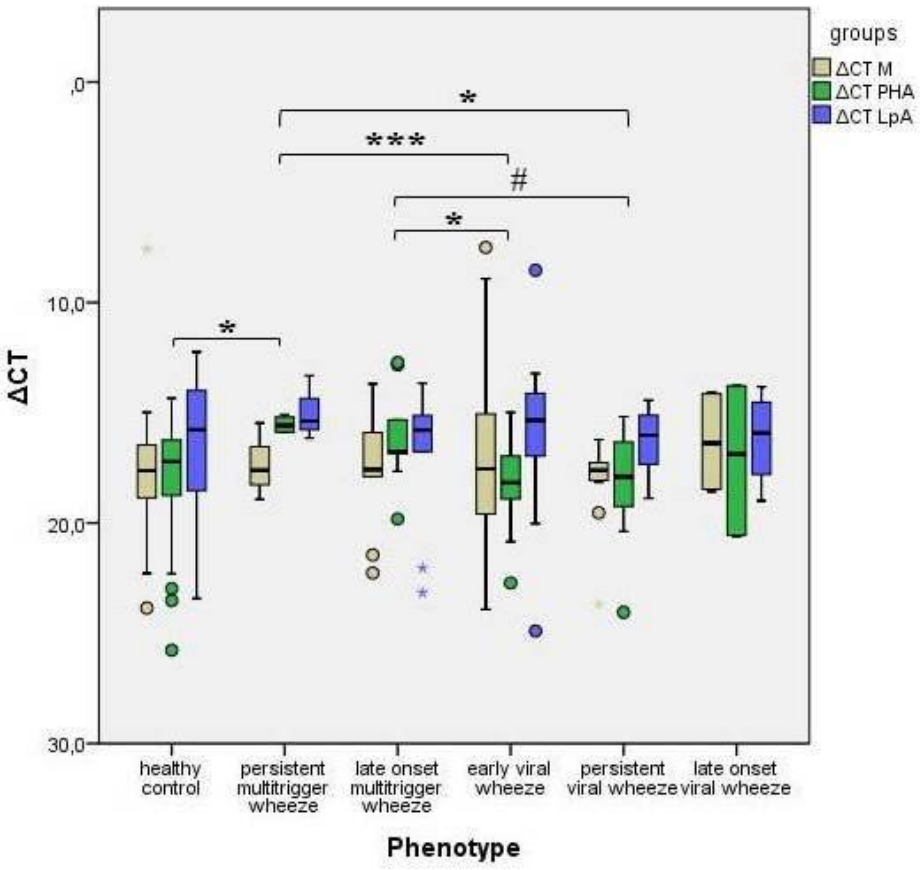


Figure 40: Difference between the detailed wheeze phenotypes for the gene expression of TLR5.
p-value: ≤0.1= # ≤ 0.05= * ; ≤ 0.01=** ,≤ 0.005=***

In the more detailed analysis, children with PMT also showed a higher gene expression following PHA stimulation. **PMT** showed significantly higher gene expression compared **with HC (p-value=0.029), PVW (p-value=0.036)) and EVW (p-value=0.004)**. **LOM** also showed a higher gene expression **in PHA compared with EVW (p-value=0.034)** and a trend to a higher gene expression compared with PVW (p-value=0.095).

Table 37: Difference between the detailed wheeze phenotypes for the gene expression of TLR5 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

TLR5		*n*	*median*	*95% CI*
HC	*M*	26	17.63	16.35;18.68
	PHA	24	17.21	16.80;19.33
	LpA	26	15.77	15.06;17.23
PMT	*M*	3	17.61	13.00;21.67
	PHA	4	15.58	14.89;16.18
	LPA	3	15.38	11.32;18.58
LOM	*M*	10	17.58	15.56;19.51
	PHA	9	16.76	14.33;17.79
	LpA	9	15.79	14.48;19.54
EVW	*M*	18	17.54	15.03;19.12
	PHA	17	18.17	17.11;19.05
	LPA	18	15.35	14.15;17.54
PVW	*M*	11	17.99	16.85;19.53
	PHA	10	16.87	12.87;21.36
	LpA	10	16.02	15.20;17.25
LOVW	*M*	6	16.39	14.23;18.44
	PHA	5	16.87	12.87;21.36
	LPA	4	15.92	12.66;19.67

4.3.7.2. TLR7

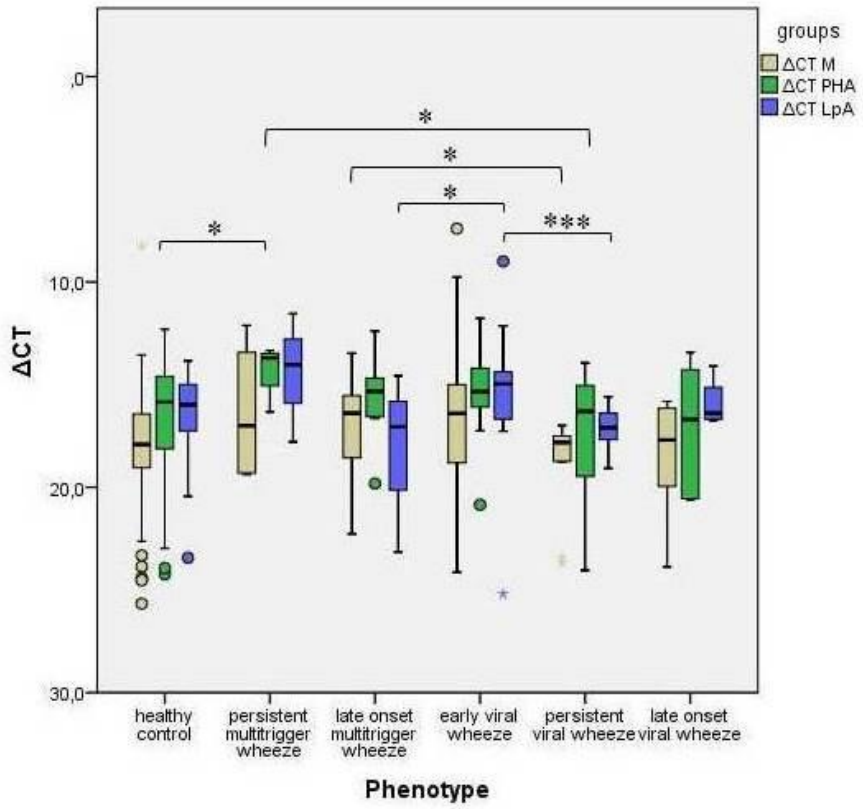


Figure 41: Difference between the detailed wheeze phenotypes for the gene expression of TLR7.
p-value: ≤0.1= # ≤0.05= * ; ≤0.01=** ,≤0.005=***

For TLR7, the more detailed analysis also showed a higher gene expression in multitrigger wheezing after PHA stimulation. **PMT** showed significantly higher gene expression compared **with HC (p-value= 0.035)** and **with PVW (p-value=0.0395).** In media, **LOM** showed significantly higher gene expression than **PVW (p-value=0.036).** After LpA stimulation, **EVW** showed higher gene expression than both **LOM (p-value=0.035) and PVW (p-value=0.004).**

Table 38: Difference between the detailed wheeze phenotypes for the gene expression of TLR7 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

TLR7		*n*	*median*	*95% CI*
HC	*M*	25	17.92	16.80;20.02
	PHA	26	15.85	15.69;18.19
	LpA	26	15.98	15.66;17.52
PMT	*M*	4	17.01	10.70;22.04
	PHA	4	13.70	12.06;16.48
	LPA	3	14.03	6.64;22.27
LOM	*M*	10	16.40	15.14;19.23
	PHA	10	15.56	14.24;16.99
	LpA	8	17.05	15.41;20.52
EVW	*M*	18	16.41	14.69;18.55
	PHA	17	15.35	14.29;16.37
	LPA	18	14.97	13.91;17.02
PVW	*M*	11	17.82	17.22;20.45
	PHA	11	16.31	15.14;19.86
	LpA	10	17.10	16.38;17.96
LOVW	*M*	6	17.70	15.37;21.69
	PHA	5	16.69	12.90;21.33
	LPA	4	16.40	13.95;17.87

4.3.7.3. RIG-I (DDX58)

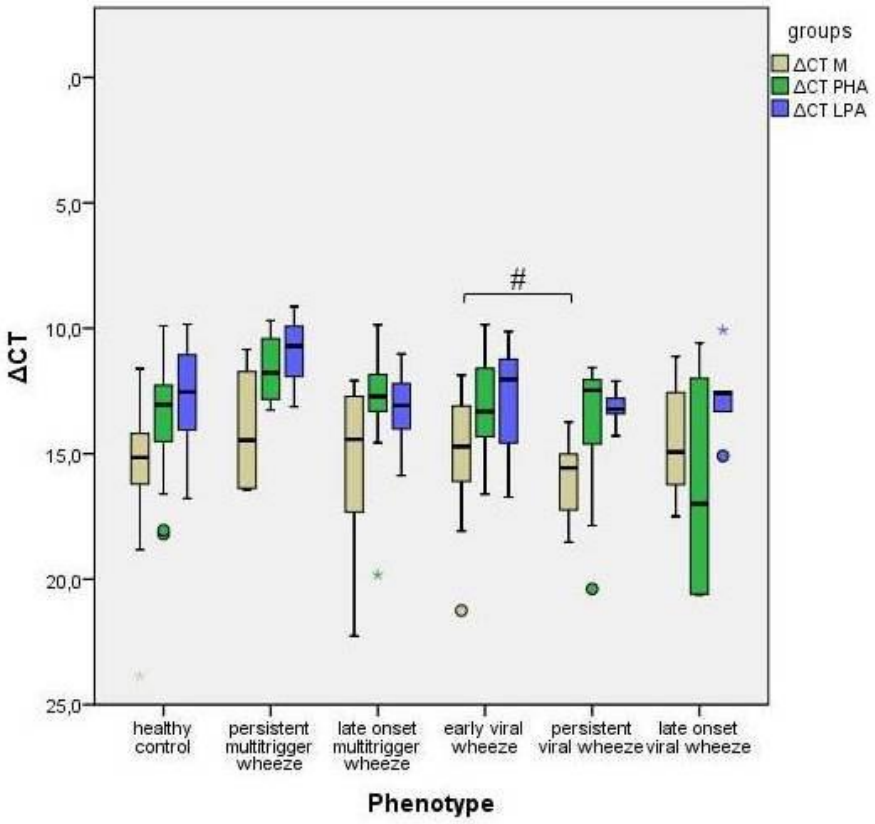


Figure 42: Difference between the detailed wheeze phenotypes for the gene expression of RIG-I
p-value: ≤0.1= # ≤0.05= * ; ≤0.01=** ,≤0.005=***

For RIG-I, the more detailed analysis revealed a higher gene expression in EVW compared with PVW (p-value= 0.055) in media.

Table 39: Difference between the detailed wheeze phenotypes for the gene expression of RIG-I on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

RIG-I		*n*	*median*	*95% CI*
HC	*M*	26	15.15	14.45;16.33
	PHA	26	13.04	12.61;14.38
	LpA	26	12.54	11.89;13.31
PMT	*M*	4	14.47	9.63;18.47
	PHA	4	11.77	9.15;14.10
	LPA	3	10.71	5.99;15.98
LOM	*M*	10	14.43	13.11;18.36
	PHA	10	12.71	11.26;15.06
	LpA	8	13.07	11.92;14.44
EVW	*M*	18	14.71	13.57;15.97
	PHA	18	13.33	12.03;13.93
	LPA	18	12.04	11.68;13.68
PVW	*M*	11	15.57	14.95;17.18
	PHA	11	12.47	12.09;15.89
	LpA	10	13.23	12.67;13.71
LOVW	*M*	6	14.93	12.08;17.01
	PHA	4	16.99	8.18;24.41
	LPA	5	12.61	10.48;14.96

4.3.7.4. MDA-5 (IFIH1)

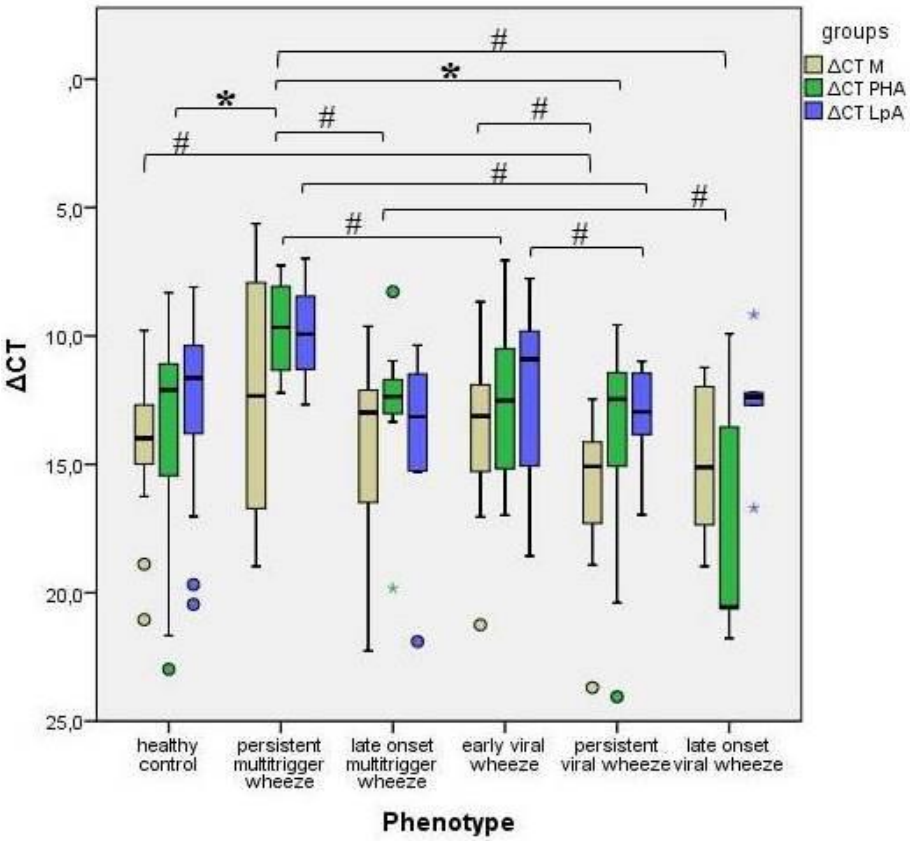


Figure 43: Difference between the detailed wheeze phenotypes for the gene expression of MDA-5.
p-value: ≤0.1= # ≤ 0.05= * ; ≤ 0.01=** ,≤ 0.005=***

For MDA-5, the more detailed analysis showed a higher gene expression for multitrigger wheezing following PHA stimulation. **PMT** showed significantly higher gene expression compared **with HC (p-value=0.044) and PVW (p-value=0.026)** and a trend to a higher gene expression than EVW (p-value=0.081) and LOVW (p-value=0.063). The gene expression in PMT was also higher than in LOM (p-value=0.054). In media, PVW showed a trend to the lowest gene expression compared with both HC (p-value=0.065) and EVW (p-value=0.082). The same could be seen after LpA stimulation, for PVW had a lower gene expression than PMT (p-value=0.077) and EVW (p-value=0.0799). Additionally, LOVW showed a lower gene expression than LOM (p-value=0.075) following PHA stimulation.

Table 40: Difference between the detailed wheeze phenotypes for the gene expression of MDA-5 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

MDA-5		*n*	*median*	*95% CI*
HC	*M*	26	13.99	13.04;15.04
	PHA	26	12.11	11.90;14.80
	LpA	26	11.64	11.25;13.72
PMT	*M*	4	12.34	3.22;21.42
	PHA	4	9.67	6.3;13.08
	LPA	3	9.93	2.79;16.93
LOM	*M*	10	12.98	11.39;17.62
	PHA	10	12.37	10.58;14.73
	LpA	9	13.15	11.05;16.49
EVW	*M*	17	13.12	12.08;15.32
	PHA	18	12.52	10.94;13.83
	LPA	18	10.91	10.42;13.63
PVW	*M*	11	15.08	13.84;18.23
	PHA	11	12.46	11.18;17.17
	LpA	10	12.96	11.68;14.33
LOVW	*M*	6	15.12	11.78;18.15
	PHA	5	20.56	10.76;23.81
	LPA	5	12.38	9.30;15.96

4.3.7.5. Mincle (CLEC4E)

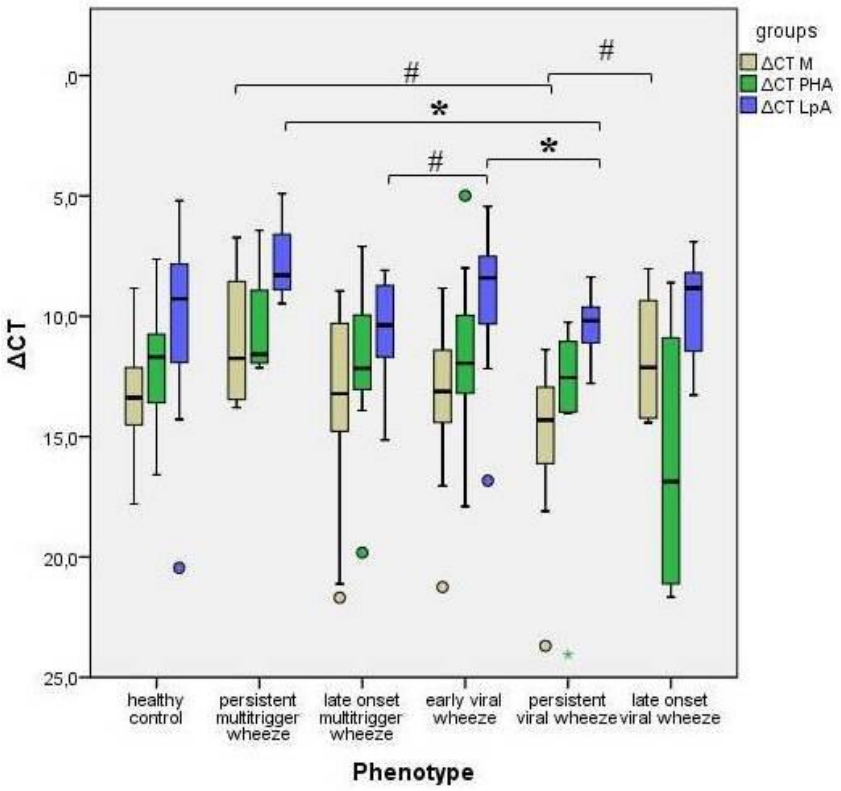


Figure 44: Difference between the detailed wheeze phenotypes for the gene expression of Mincle.
p-value: ≤0.1= # ≤0.05= * ; ≤0.01=** ,≤0.005=***

For Mincle, the detailed analysis showed a lower gene expression in PVW in media compared with PMT (p-value= 0.056) and LOVW (p-value=0.078). This downregulation was significant for **PVW** after LpA stimulation compared **with PMT (p-value =0.028)** and **with EVW (p-value=0.047)**. Additionally, LOM showed a trend towards a lower gene expression than EVW (p-value=0.068).

Table 41: Difference between the detailed wheeze phenotypes for the gene expression of Mincle on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

Mincle		*n*	*median*	*95% CI*
HC	*M*	25	13.38	12.59;14.17
	PHA	25	11.69	11.11;13.08
	LpA	26	9.28	8.68;11.32
PMT	*M*	4	11.76	5.90;16.12
	PHA	4	11.59	6.16;14.71
	LPA	3	8.3	1.65;13.47
LOM	*M*	10	13.68	10.69;16.99
	PHA	10	12.17	9.48;14.47
	LpA	9	10.37	8.85;12.32
EVW	*M*	17	13.12	11.60;14.65
	PHA	18	12.55	10.52;16.21
	LPA	18	8.41	7.71;10.43
PVW	*M*	11	14.31	12.65;17.46
	PHA	10	12.15	10.17;16.65
	LpA	10	10.19	9.32;11.48
LOVW	*M*	6	12.13	9.01;14.42
	PHA	4	16.87	6.13;25.87
	LPA	5	8.83	6.52;12.93

4.3.7.6. Dectin1 (CLEC7A)

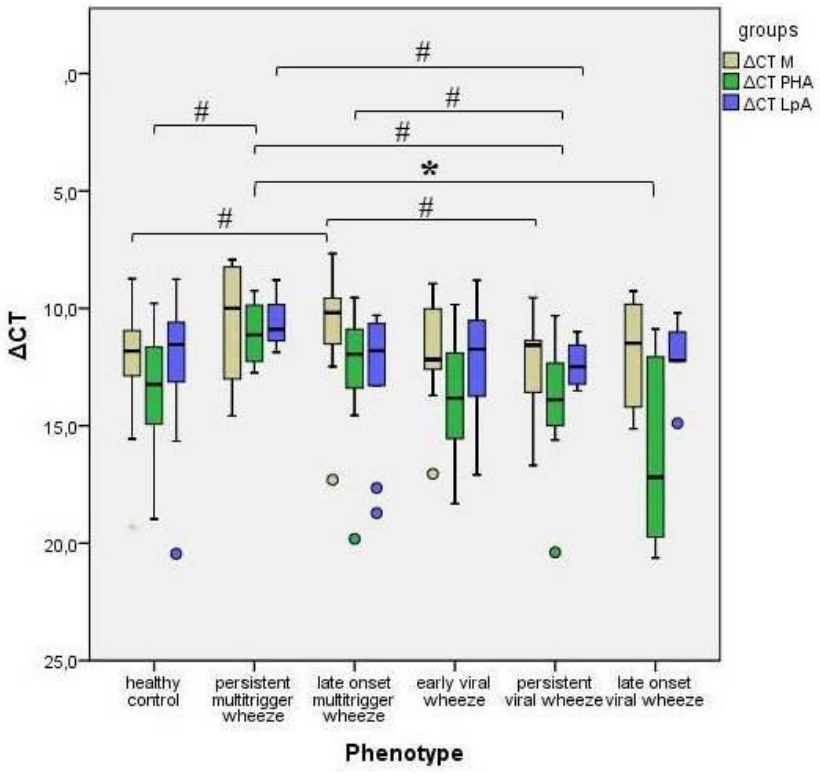


Figure 45: Difference between the detailed wheeze phenotypes for the gene expression of Dectin1.
p-value: ≤0.1= # ≤0.05= * ; ≤0.01=** ,≤0.005=***

For Dectin1, the detailed analysis showed a higher gene expression in multitrigger wheezing. In media, LOM showed a trend to a higher gene expression than PVW (p-value=0.095) and HC (p-value=0.086). After PHA stimulation, **PMT** showed significantly higher gene expression **than PVW (p-value=0.043)** and higher gene expression than EVW (p-value=0.053) and HC (p-value=0.062). LOM also showed a trend towards a higher gene expression than PVW (p-value= 0.098). After LpA stimulation, PMT showed a higher gene expression than PVW (p-value=0.078).

Table 42: Difference between the detailed wheeze phenotypes for the gene expression of Dectin1 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

Dectin1		*n*	*median*	*95% CI*
HC	*M*	26	11.82	11.28;13.07
	PHA	25	13.23	12.55;14.50
	LpA	26	11.54	11.07;13.05
PMT	*M*	4	10.56	5.77;15.48
	PHA	4	11.14	8.64;13.49
	LPA	3	10.89	6.62;14.42
LOM	*M*	9	10.19	8.97;13.14
	PHA	10	11.95	10.49;14.73
	LpA	9	11.80	10.55;15.37
EVW	*M*	17	12.18	10.67;12.78
	PHA	18	13.83	12.56;14.89
	LPA	18	11.75	11.09;13.23
PVW	*M*	11	11.57	11.04;13.76
	PHA	11	13.89	12.26;15.81
	LpA	10	12.49	11.67;13.06
LOVW	*M*	6	11.87	9.43;14.37
	PHA	5	17.19	10.60;21.60
	LPA	5	12.22	9.91;14.32

4.3.7.7. Dectin2 (CLEC6A)

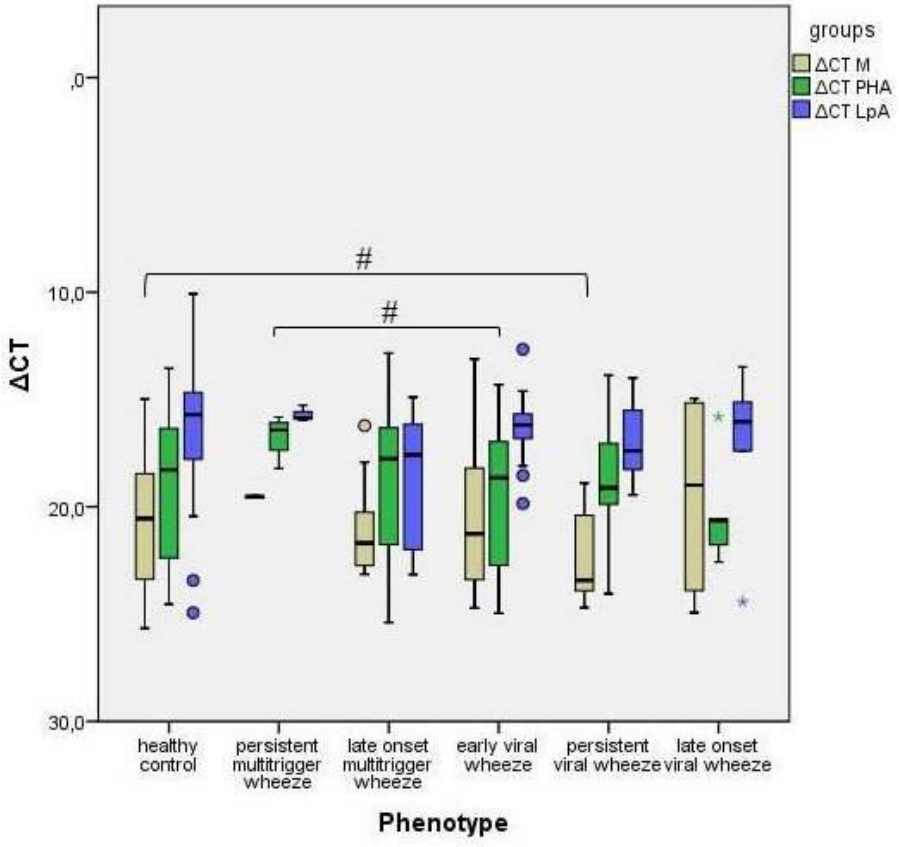


Figure 46: Difference between the detailed wheeze phenotypes for the gene expression of Dectin2.
p-value: ≤0.1= # ≤ 0.05= * ; ≤ 0.01=** ,≤ 0.005=***

For Dectin2, the detailed analysis showed a trend to a lower gene expression in media in PVW compared with HC (p-value=0.099) and after PHA stimulation in EVW compared with PMT (p-value=0.081). These findings must be seen in context with the high non-detection rate meaning a limited informative value.

Table 43: Difference between the detailed wheeze phenotypes for the gene expression of Dectin2 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

Dectin2		*n*	*median*	*95% CI*
HC	*M*	24	20.55	19.27;21.85
	PHA	24	18.28	17.59;20.52
	LpA	25	15.71	15.15;17.84
PMT	*M*	2	19.54	18.96;20.11
	PHA	4	16.42	15.06;18.38
	LPA	3	15.86	14.76;16.64
LOM	*M*	9	21.69	19.10;22.80
	PHA	8	17.77	15.29;22.20
	LpA	9	17.58	15.95;20.82
EVW	*M*	18	21.26	18.94;22.19
	PHA	18	18.65	17.85;21.19
	LPA	17	16.19	15.54;17.23
PVW	*M*	11	23.43	20.89;23.75
	PHA	11	19.12	16.88;20.92
	LpA	10	17.40	15.69;18.29
LOVW	*M*	6	18.99	15.04;23.93
	PHA	5	20.63	17.00;23.53
	LPA	5	16.04	12.04;22.54

4.3.7.8. LMP2

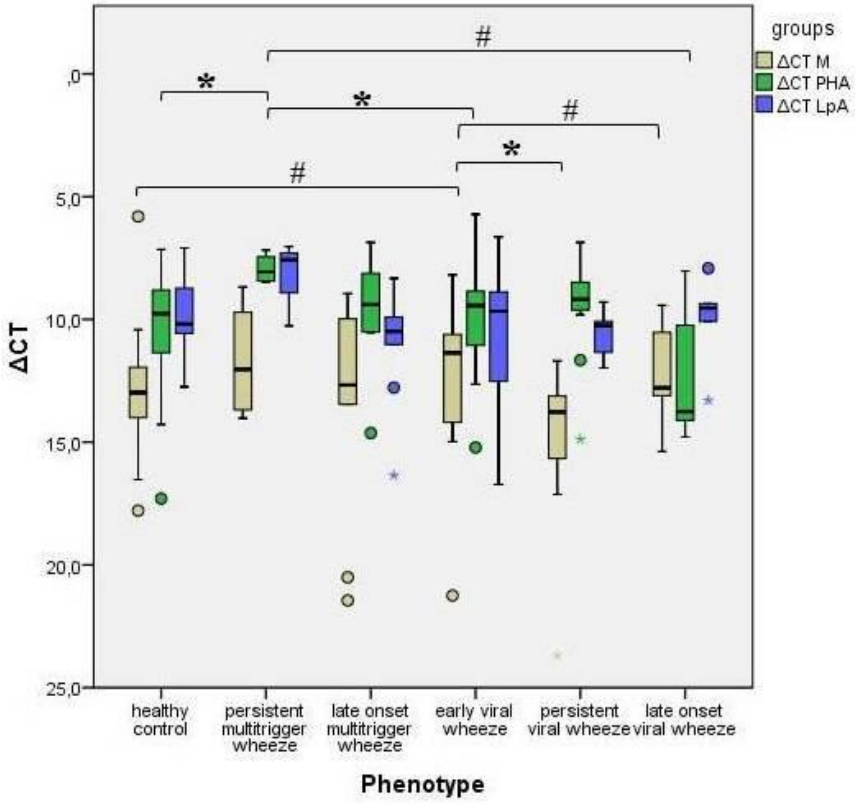


Figure 47: Difference between the detailed wheeze phenotypes for the gene expression of LMP2.
p-value: ≤0.1= # ≤ 0.05= * ; ≤ 0.01=** ,≤ 0.005=***

For LMP2, the detailed analysis showed a significantly lower gene expression in media for **PVW** compared **with EVW (p-value=0.014)** and a trend to a lower gene expression compared with HC (p-value=0.0697) and with LOVW (p-value=0.078). After PHA stimulation, there was a significantly higher gene expression in **PMT** than in **HC (p-value=0.016) and in EVW (p-value=0.033)**. There also was a trend to a higher gene expression compared with LOVW (p-value=0.063).

Table 44: Difference between the detailed wheeze phenotypes for the gene expression of LMP2 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

LMP2		*n*	*median*	*95% CI*
HC	*M*	26	12.98	12.083.90
	PHA	26	9.76	9.36;11.24
	LpA	26	10.19	9.25;10.64
PMT	*M*	4	12.04	7.78;15.61
	PHA	4	8.06	6.97;8.92
	LPA	3	7.58	4.00;12.58
LOM	*M*	10	12.68	10.10;16.40
	PHA	10	9.39	8.10;11.10
	LpA	9	10.49	9.14;12.79
EVW	*M*	18	11.37	10.92;13.83
	PHA	18	9.45	8.81;10.94
	LPA	18	9.67	9.13;11.73
PVW	*M*	11	13.78	12.67;17.13
	PHA	11	9.18	8.04;10.96
	LpA	10	10.25	9.97;11.19
LOVW	*M*	6	12.79	10.14;14.54
	PHA	5	13.76	8.56;15.81
	LPA	5	9.54	7.57;12.51

4.3.7.9. LMP7

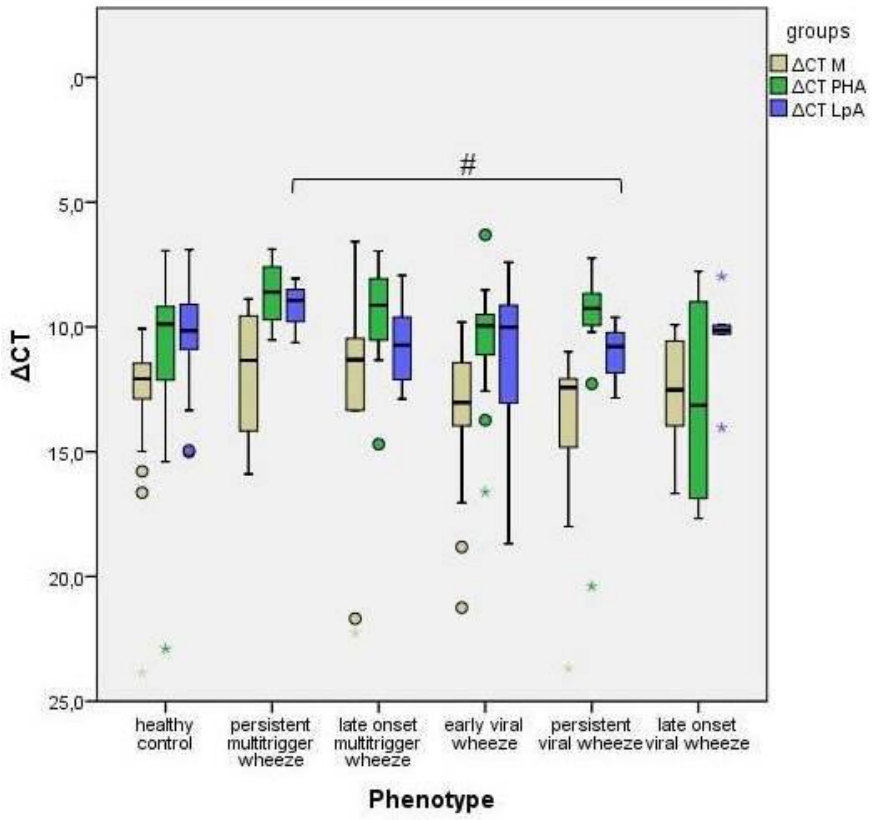


Figure 48: Difference between the detailed wheeze phenotypes for the gene expression of LMP7.
p-value: ≤0.1= # ≤ 0.05= * ; ≤ 0.01=** ,≤ 0.005=***

For LMP7, the detailed analysis showed a trend to a higher gene expression following LpA stimulation in PMT than in PVW (p-value=0.077).

Table 45: Difference between the detailed wheeze phenotypes for the gene expression of LMP7 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

LMP7		*n*	*median*	*95% CI*
HC	*M*	26	12.08	11.58;13.87
	PHA	26	9.89	9.51;12.13
	LpA	25	10.15	9.43;11.12
PMT	*M*	4	11.34	6.99;16.74
	PHA	4	8.61	6.26;11.05
	LPA	3	8.94	5.97;12.45
LOM	*M*	10	11.32	8.97;16.50
	PHA	10	9.14	8.04;11.21
	LpA	8	10.73	9.33;12.10
EVW	*M*	18	13.04	11.81;14.84
	PHA	17	9.95	9.39;11.76
	LPA	17	10.01	9.38;12.56
PVW	*M*	11	12.43	11.71;16.73
	PHA	11	9.26	7.76;12.64
	LpA	10	10.79	10.24;11.82
LOVW	*M*	6	12.52	10.10;15.28
	PHA	4	13.14	5.45;20.41
	LPA	5	10.14	7.74;13.21

4.3.7.10. NLRP3

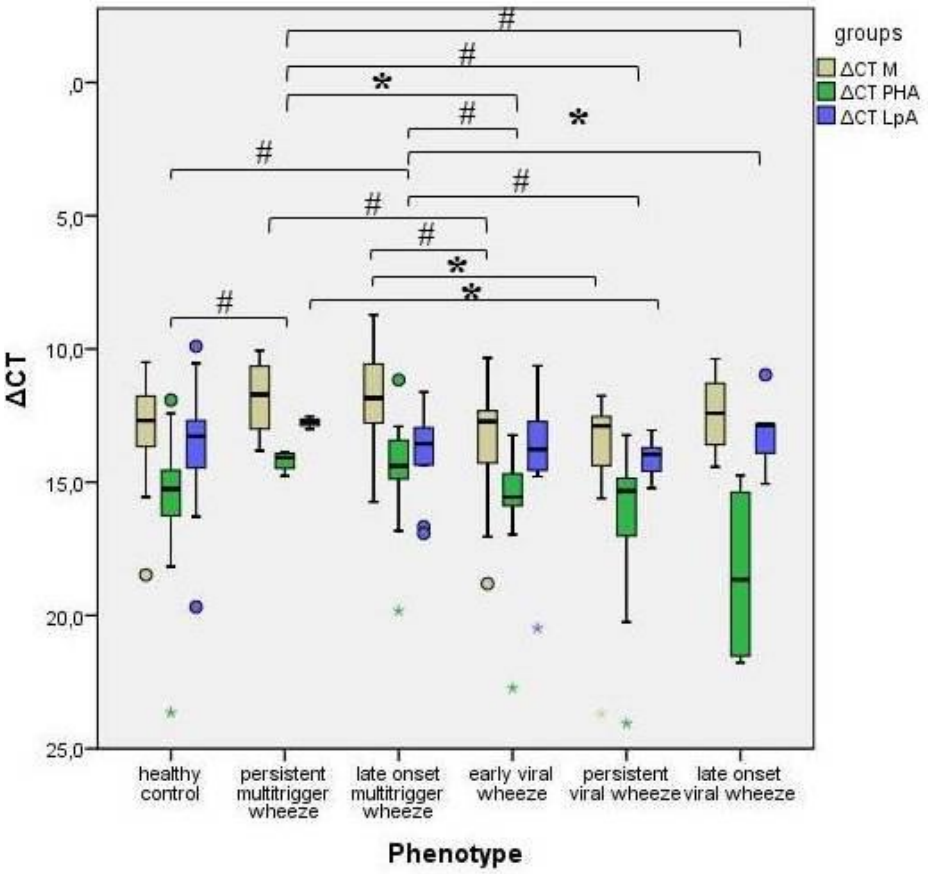


Figure 49: Difference between the detailed wheeze phenotypes for the gene expression of NLRP3.
p-value: ≤0.1= # ≤ 0.05= * ; ≤ 0.01=** ,≤ 0.005=***

For NLRP3, the detailed analysis showed a higher gene expression in multitrigger wheezing over all stimulation conditions. In media, **LOM** showed a higher gene expression compared **with PVW (p-value=0.0486)** and with EVW (p-value=0.058). PMT showed a trend to a higher gene expression than EVW (p-value=0.097). After PHA stimulation, both **PMT** and LOM showed a higher gene expression **than HC (p-value=0.052** and p- value=0.063), **EVW (p- value=0.024** and p- value=0.066), PVW (p-value=0.058 and p- value=0.072) and LOVW (p- value=0.057 and p- value=0.036). After LpA stimulation, **PMT** showed a higher gene expression than **PVW (p- value=0.014)**.

Table 46: Difference between the detailed wheeze phenotypes for the gene expression of NLRP3 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

NLRP3		*n*	*median*	*95% CI*
HC	*M*	26	12.69	12.21;13.57
	PHA	26	15.26	14.74;16.54
	LpA	26	13.28	12.78;14.38
PMT	*M*	4	11.71	9.30;14.35
	PHA	4	14.08	13.57;14.83
	LPA	3	12.74	12.16;13.36
LOM	*M*	10	11.85	10.31;13.25
	PHA	10	14.40	12.99;16.32
	LpA	9	13.56	12.57;15.32
EVW	*M*	18	12.73	12.43;14.53
	PHA	17	15.56	14.65;16.74
	LPA	18	13.77	12.87;14.83
PVW	*M*	11	12.89	11.93;16.44
	PHA	11	15.33	14.46;1.64
	LpA	10	13.96	13.65;14.56
LOVW	*M*	6	12.42	10.86;13.97
	PHA	4	18.65	12.74;24.16
	LPA	5	12.85	11.25;15.01

4.3.7.11. Casp1

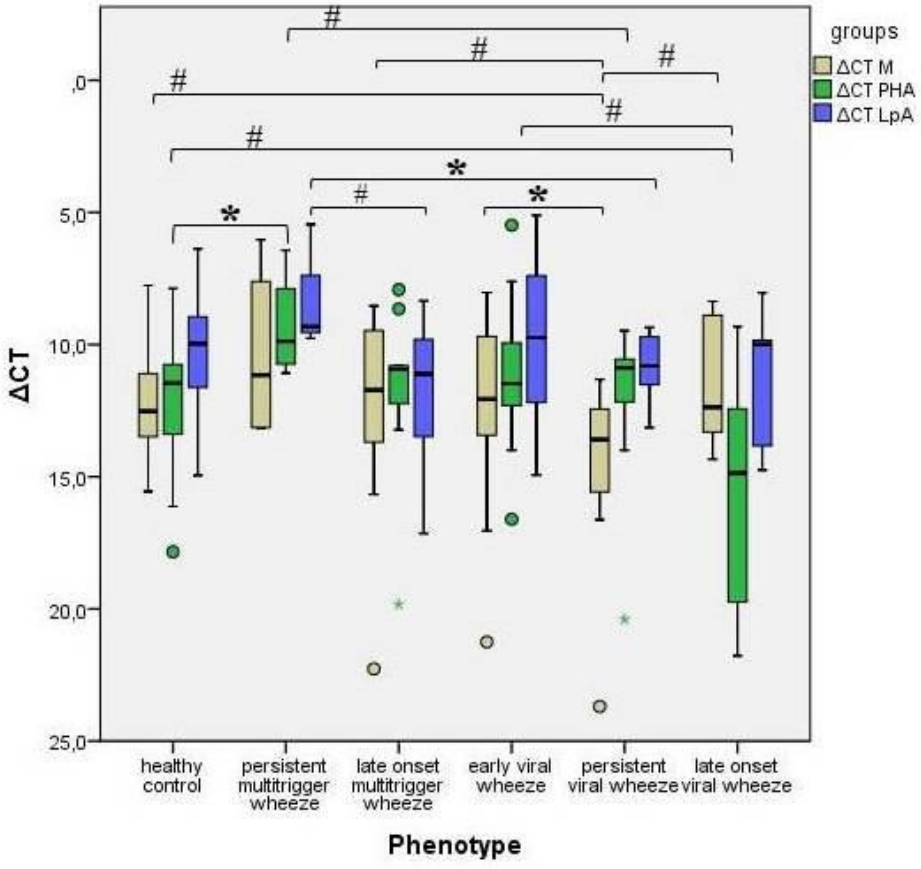


Figure 50: Difference between the detailed wheeze phenotypes for the gene expression of Casp1.
p-value: ≤0.1= # ≤0.05= * ; ≤0.01=** ,≤0.005=***

For Casp1, the detailed analysis showed a lower gene expression in **PVW** in media compared with HC (p-value=0.074), **EVW (p-value= 0.041)**, LOVW (p-value= 0.097) and LOM (p-value= 0.084). After PHA stimulation, **PMT** showed a higher gene expression **than HC (p-value=0.022)** and PVW (p-value= 0.078). LOVW showed a lower gene expression compared with HC (p-value=0.091) and EVW (p-value=0.055). After LpA stimulation, **PMT** showed a higher gene expression **than PVW (p-value=0.049)** and than LOM (p-value=0.063).

Table 47: Difference between the detailed wheeze phenotypes for the gene expression of Casp1 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

CASP1		*n*	*median*	*95% CI*
HC	*M*	25	12.52	11.67;13.20
	PHA	26	11.46	10.95;12.85
	LpA	26	9.96	9.40;11.20
PMT	*M*	4	11.16	4.90;15.85
	PHA	4	9.88	6.05;12.58
	LPA	3	9.32	2.29;14.07
LOM	*M*	10	11.72	9.51;15.41
	PHA	10	10.94	9.37;14.01
	LpA	9	11.11	9.71;13.88
EVW	*M*	18	12.07	10.56;13.87
	PHA	18	11.48	9.82;12.37
	LPA	18	9.74	8.48;11.13
PVW	*M*	11	13.59	12.21;16.87
	PHA	11	10.88	9.95;14.06
	LpA	10	10.81	10.05;11.75
LOVW	*M*	6	12.37	9.05;14.16
	PHA	5	14.86	9.26;21.99
	LPA	5	9.99	7.74;14.84

4.3.7.12. IL-1R1

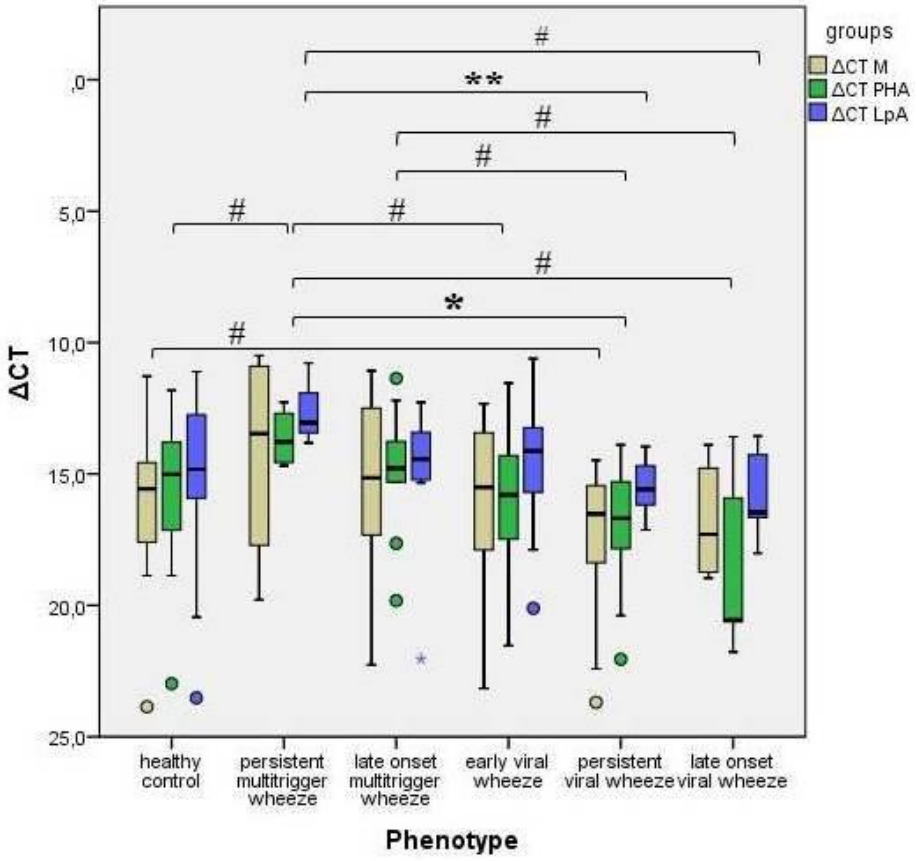


Figure 51: Difference between the detailed wheeze phenotypes for the gene expression of IL1R1.
p-value: ≤0.1= # ≤ 0.05= * ; ≤ 0.01=** ,≤ 0.005=***

For IL-1R1, the detailed analysis showed a higher gene expression in multitrigger wheezing following PHA stimulation and LpA stimulation. In PHA, **PMT** showed higher gene expression **than PVW (p-value=0.017)**, LOVW (p-value=0 .063), EVW (p-value= 0.074) and HC (p-value=0.082). LOM showed higher gene expression than PVW (p-value=0.061) and LOVW (p-value=0.075). After LpA stimulation, **PMT** showed significantly higher gene expression **than PVW (p-value=0.007)** and a trend to a higher gene expression than LOVW (p-value=0.071). In media, PVW showed a trend to a lower gene expression than HC (p-value=0.074).

Table 48: Difference between the detailed wheeze phenotypes for the gene expression of IL-1R1 on ΔC_T level within the different stimuli (unstimulated; PHA and LpA) and number of available samples (n)

IL-1R1		*n*	*median*	*95% CI*
HC	*M*	25	15.57	14.77;16.93
	PHA	26	15.02	14.63;16.58
	LpA	26	14.82	13.78;16.13
PMT	*M*	4	13.47	7.46;21.15
	PHA	4	13.78	11.82;15.44
	LPA	3	13.05	8.62;16.48
LOM	*M*	10	15.15	13.00;18.46
	PHA	10	14.79	13.14;16.64
	LpA	8	14.44	12.54;17.58
EVW	*M*	18	15.51	14.40;17.48
	PHA	18	15.80	14.67;17.00
	LPA	18	14.13	13.44;15.65
PVW	*M*	11	16.52	15.63;19.68
	PHA	11	16.69	15.20;18.60
	LpA	10	15.59	14.69;16.22
LOVW	*M*	6	17.30	14.59;19.06
	PHA	5	20.56	14.09;22.90
	LPA	5	16.46	13.52;18.07

4.3.8. Gene expression patterns considering temporal aspects of wheezing symptoms

Table 49 shows a general overview of gene expression patterns for the more detailed phenotypes. Significant results are marked green (p-value <0,05), trends are marked orange (p-value < 0,1).

Table 49.1: Overview of gene expression patterns for more detailed phenotypes

	TLR5			TLR7			RIG-I			MDA-5		
	media	PHA	LpA	media	PHA	LpA	media	PHA	LpA	media	PHA	LpA
PMT vs PVW	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑
PMT vs LOVW	PMT ↓	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑
PMT vs EVW	PMT ↓	PMT ↑	PMT ↓	PMT ↓	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑
PMT vs HC	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑
PMT vs LOM	PMT ↓	PMT ↑	PMT ↑	PMT ↓	PMT ↑	PMT ↑	PMT ↓	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑
LOM vs PVW	LOM ↑	LOM ↑	LOM ↑	LOM ↑	LOM ↑	LOM ↑	LOM ↑	LOM ↓	LOM ↑	LOM ↑	LOM ↑	LOM ↓
LOM vs LOVW	LOM ↓	LOM ↑	LOM ↑	LOM ↑	LOM ↑	LOM ↓	LOM ↑	LOM ↑	LOM ↓	LOM ↑	LOM ↑	LOM ↓
LOM vs EVW	LOM ↓	LOM ↑	LOM ↓	LOM ↑	LOM ↓	LOM ↓	LOM ↑	LOM ↑	LOM ↓	LOM ↑	LOM ↑	LOM ↓
LOM vs HC	LOM ↑	LOM ↓	LOM ↑	LOM ↑	LOM ↑	LOM ↓	LOM ↑	LOM ↑	LOM ↓	LOM ↑	LOM	LOM ↓
PVW vs LOVW	PVW ↓	PVW ↔	PVW ↓	PVW ↓	PVW ↑	PVW ↓	PVW ↓	PVW ↑	PVW ↓	PVW ↑	PVW ↑	PVW ↓
PVW vs EVW	PVW ↓	PVW ↑	PVW ↓	PVW ↓	PVW ↓	PVW ↓	PVW ↓	PVW ↑	PVW ↓	PVW ↓	PVW ↑	PVW ↓
PVW vs HC	PVW ↓	PVW ↑	PVW ↓	PVW ↑	PVW ↓	PVW ↓	PVW ↓	PVW ↑	PVW ↓	PVW ↓	PVW ↓	PVW ↓
LOVW vs EVW	LOV ↑	LOV ↑	LOV ↓	LOV ↓	LOV ↓	LOV ↓	LOV ↓	LOV ↓	LOV ↓	LOV ↓	LOV ↓	LOV ↓
LOVW vs HC	LOV ↑	LOV ↑	LOV ↓	LOV ↑	LOV ↓	LOV ↓	LOV ↑	LOV ↓	LOV ↓	LOV ↓	LOV ↓	LOV ↓
EVW vs HC	EV W ↑	EV W ↓	EV W ↑	EV W ↑	EV W↑	EV W ↑	EV W ↑	EV W ↓	EV W ↑	EV W ↑	EV W ↓	EW V ↑

Table 48.2: Overview of gene expression patterns for more detailed phenotypes

	Mincle			Dectin1			Dectin2			LMP2		
	media	PHA	LpA	media	PHA	LpA	media	PHA	LpA	media	PHA	LpA
PMT vs PVW	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑
PMT vs LOVW	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↓	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑
PMT vs EVW	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↓	PMT ↑	PMT ↑
PMT vs HC	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↓	PMT ↑	PMT ↑	PMT ↑
PMT vs LOM	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↓	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑
LOM vs PVW	LOM ↑	LOM ↓	LOM ↓	LOM ↑	LOM ↑	LOM ↑	LOM ↑	LOM ↑	LOM ↓	LOM ↑	LOM ↓	LOM ↓
LOM vs LOVW	LOM ↓	LOM ↑	LOM ↓	LOM ↑	LOM ↑	LOM ↑	LOM ↓	LOM ↑	LOM ↓	LOM ↑	LOM ↑	LOM ↓
LOM vs EVW	LOM ↓	LOM ↑	LOM ↓	LOM ↑	LOM ↑	LOM ↓	LOM ↓	LOM ↑	LOM ↓	LOM ↓	LOM ↑	LOM ↓
LOM vs HC	LOM ↓	LOM ↓	LOM ↓	LOM ↑	LOM ↑	LOM ↓	LOM ↓	LOM ↓	LOM ↓	LOM ↑	LOM ↑	LOM ↓
PVW vs LOVW	PVW ↓	PVW ↑	PVW ↓	PVW ↑	PVW ↑	PVW ↓	PVW ↓	PVW ↑	PVW ↓	PVW ↓	PVW ↑	PVW ↓
PVW vs EVW	PVW ↓	PVW ↑	PVW ↓	PVW ↑	PVW ↓	PVW ↓	PVW ↓	PVW ↓	PVW ↓	PVW ↓	PVW ↑	PVW ↓
PVW vs HC	PVW ↓	PVW ↓	PVW ↓	PVW ↑	PVW ↓	PVW ↓	PVW ↓	PVW ↓	PVW ↓	PVW ↓	PVW ↑	PVW ↓
LOVW vs EVW	LOV ↑	LOV ↓	LOV ↓	LOV ↑	LOV ↓	LOV ↓	LOV ↑	LOV ↓	LOV ↑	LOV ↓	LOV ↓	LOV ↑
LOVW vs HC	LOV ↑	LOV ↓	LOV ↑	LOV ↓	LOV ↓	LOV ↓	LOV ↑	LOV ↓	LOV ↓	LOV ↑	LOV ↓	LOV ↑
EVW vs HC	EV W ↑	EV W ↓	EV W ↑	EV W ↓	EV W ↓	EV W ↓	EV W ↓	EV W ↓	EV W ↓	EV W ↑	EV W ↑	EV W ↑

Table 48.3: Overview of gene expression patterns for more detailed phenotypes

	LMP7			NLRP3			Casp1			IL-1R1		
	media	PHA	LpA	media	PHA	LpA	media	PHA	LpA	media	PHA	LpA
PMT vs PVW	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑
PMT vs LOVW	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑
PMT vs EVW	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑
PMT vs HC	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑
PMT vs LOM	PMT ↓	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑	PMT ↑
LOM vs PVW	LOM ↑	LOM ↑	LOM ↑	LOM ↑	LOM ↑	LOM ↑	LOM ↑	LOM ↓	LOM ↑	LOM ↑	LOM ↑	LOM ↑
LOM vs LOVW	LOM ↑	LOM ↑	LOM ↓	LOM ↑	LOM ↑	LOM ↓	LOM ↑	LOM ↑	LOM ↓	LOM ↑	LOM ↑	LOM ↑
LOM vs EVW	LOM ↑	LOM ↑	LOM ↓	LOM ↑	LOM ↑	LOM ↑	LOM ↑	LOM ↑	LOM ↓	LOM ↑	LOM ↑	LOM ↓
LOM vs HC	LOM ↑	LOM ↑	LOM ↓	LOM ↑	LOM ↑	LOM ↓	LOM ↑	LOM ↑	LOM ↓	LOM ↑	LOM ↑	LOM ↑
PVW vs LOVW	PVW ↑	PVW ↑	PVW ↓	PVW ↓	PVW ↑	PVW ↓	PVW ↓	PVW ↑	PVW ↑	PVW ↑	PVW ↑	PVW ↑
PVW vs EVW	PVW ↑	PVW ↑	PVW ↓	PVW ↓	PVW ↑	PVW ↓	PVW ↓	PVW ↑	PVW ↓	PVW ↓	PVW ↓	PVW ↓
PVW vs HC	PVW ↓	PVW ↑	PVW ↓	PVW ↓	PVW ↓	PVW ↓	PVW ↓	PVW ↑	PVW ↓	PVW ↓	PVW ↓	PVW ↓
LOVW vs EVW	LOV ↑	LOV ↓	LOV ↓	LOV ↑	LOV ↓	LOV ↑	LOV ↓	LOV ↓	LOV ↓	LOV ↓	LOV ↓	LOV ↓
LOVW vs HC	LOV ↓	LOV ↓	LOV ↑	LOV ↑	LOV ↓	LOV ↑	LOV ↑	LOV ↓	LOV ↓	LOV ↓	LOV ↓	LOV ↓
EVW vs HC	EV W ↓	EV W ↓	EV W ↑	EV W ↓	EV W ↓	EV W ↓	EV W ↑	EV W ↓	EV W ↑	EV W ↑	EV W ↓	EV W ↑

4.3.9. Added value of the more detailed phenotype analysis

The more detailed phenotype analysis revealed that children with persistent symptoms most strongly contributed to the found gene expression differences.
This effect could be seen especially for persistent multitrigger wheeze. After PHA stimulation, children with persistent multitrigger wheeze showed a significant upregulation of the gene expression of MDA-5, LMP2, NLRP3, Casp1 and IL-1R1. For Mincle, NLRP3, Casp1 and IL-1R1, this was also detectable after LpA stimulation.
In parallel, it was children with persistent viral wheeze who showed the most significant reduction in gene expression among all children with viral wheeze. This downregulation was most strongly detectable for MDA-5, LMP2 and Casp1 in unstimulated condition and for TLR7 and Mincle after LpA stimulation.

In summary, the more detailed phenotype analysis allowed a more in-depth insight into the importance of the temporal aspect of the wheezing symptoms. However, these findings have to be interpreted with caution as the sample size decreases within the subgroups.

5. DISCUSSION

5.1.Main findings

(1) The expression of most of the tested genes of the innate immune system, the inflammasome and the immunoproteasome increased significantly after stimulation of cord blood mononuclear cells with PHA and LpA. Interestingly, Dectin1 and NLRP3 showed a significant downregulation after stimulation with PHA.

(2) Said genes differed significantly between asymptomatic newborns according to their subsequent wheeze phenotype. Healthy controls showed a different gene expression compared to children with multitrigger wheeze and children with persistent or late onset viral wheeze but not compared to children with early viral wheeze.

(3a) Within the phenotype comparison, children with multitrigger wheeze showed the highest gene expression overall and children with viral wheeze the lowest compared with the other phenotypes. Interestingly, the gene expression of healthy controls was ranked in between the symptomatic phenotypes indicating that subsequently healthy controls might have a more controlled immune balance than children with symptoms in the first years of life.

(3b) The more detailed phenotype analysis including a temporal pattern of wheeze showed that persistent multitrigger wheeze most strongly induced the upregulation of gene expression in children with multitrigger wheeze.

(4) Multitrigger wheeze and viral wheeze differed most clearly with increased gene expression of TLR5, Dectin1, NLRP3 and IL-1R1 in children with multitrigger wheeze compared to a decreased gene expression of TLR7, MDA-5 and IL-1R1 in children with viral wheeze. This indicated different disease entities, characterized by distinct immune regulation, of the wheeze phenotypes.

(5) Some pathways, especially the NLRP3/IL-1R1 axis, are already regulated differently at birth which may implicate a genetic or epigenetic component for the different phenotypes, especially persistent multitrigger wheeze.

5.1.1. Differences in gene expression and phenotype characteristics

5.1.1.1. Multitrigger wheeze is characterized by an upregulation of gene expression

The group comparison of the wheeze phenotypes revealed that children classified as multitrigger wheezers showed an overall upregulation of the examined genes. Especially genes related to the inflammasome/IL-1R1 axis were consistently increased in children with multitrigger wheeze with this effect being significant for NLRP3 and IL-1R1. It has been shown previously that the NLRP3 inflammasome together with Casp1 is upregulated in neutrophilic asthma in adults [71]. In this project, an upregulation of these genes was already detectable at birth (see Figure 52).
At birth, children with subsequent multitrigger wheeze showed the highest gene expression after PHA stimulation. This was not only shown for the inflammasome/IL-1R1 axis but also for several genes encoding for PPRs (TLR5, TLR7, and Dectin1).
This increased expression could play an important role for the development of wheeze symptoms during immune maturation. The impact of other PPRs on inflammation and asthma development have already been shown [53, 76].
The upregulation of gene expression after PHA stimulation at birth in children with future multitrigger wheeze highlights the strong susceptibility of children with multitrigger wheeze to possible triggers. This may be a hint of an immune imbalance or potential deficiency in control mechanisms which leads to exuberant activation already at a time when the child is clinically asymptomatic. Subsequently, these children develop symptoms of wheeze in pre-school age.
In summary, multitrigger wheeze was characterized by an upregulation of gene expression encoding for PRRs, inflammasome and the IL-1R1 axis together with upregulated immunoproteasome genes. These results may point out a genetic component for the development of a multitrigger wheeze phenotype in childhood.

5.1.1.2. Viral wheeze is characterized by a downregulation of gene expression

In contrast, children with future persistent or late onset viral wheeze presented an overall downregulation of gene expression at birth compared with the other phenotypes.
Interestingly, the downregulation was most strongly observed after stimulation with either PHA or LpA. After PHA stimulation, IL-1R1 was significantly downregulated compared with healthy controls. The gene expression only showed a trend towards a downregulation under unstimulated conditions (MDA-5 and IL-1R1) which could be due to the limited sample size. Additionally, there was a downregulation of PRRs (TLR7, MDA-5, and Mincle) detectable after LpA stimulation.
This might highlight that the imbalance of the immune system of those children could be triggered by environmental factors acting as a ‘second hit’.
This may indicate that children presenting with persistent and/or late onset viral wheeze might be unable to react adequately to immune stimuli due to deficiency in specific innate immune system pathways. This can result in an inefficiency to respond to viral infections

leading to a longer and more severe infection period presenting with clinical symptoms of wheeze. In accordance with this finding, it has been shown that among asthmatic children with virus-triggered exacerbations those prone to viral re-infection show an impaired anti-viral response with altered PRR function [77]. Childhood viral infections with persistent wheeze are known to be a risk factor for asthma development [78].
These findings indicate the existence of a host factor explaining differences of symptom features such as vulnerability to or duration of wheeze. This is supported by another study that showed that the duration of wheeze symptoms during an infection is independent of the microbial trigger [79]. In summary, symptoms of children with persistent and/or late onset viral wheeze could be triggered by environmental factors revealing the deficiency of specific innate immune pathways that were detectable already at birth. This is in line with findings from Spycher et al. that indicate different disease entities for children with multitrigger wheeze and children with early viral wheeze [80].

5.1.1.3. Early viral wheeze and healthy controls feature a similar gene expression pattern

There was no statistically significant difference between early viral wheeze and healthy controls detectable. This may indicate that children with future early viral wheeze with no complications such as hospitalization and healthy children have similar immune regulation at birth. The children with early viral wheeze may however react with mild self-limiting symptoms. Therefore, children with early viral wheeze and balanced immune regulation at birth may have a rather small potential risk for asthma development. This finding is supported by other studies that distinguish between children with an elevated asthma risk and children with transient early wheeze [81, 82]. In addition, it has been shown that children wheezing only within the first 3 years of life were as unlikely to show wheezing symptoms later in life as healthy controls [83, 84]. Thus, for this group of children it may actually be very informative to have early life immune regulation data available. Intense treatment could be potentially avoided, and conversely rather be applied to children with future multitrigger wheeze.
Healthy controls, defined by no symptoms at any age of follow-up, showed a gene expression in range between children with multitrigger wheeze and children with viral wheeze. This finding might indicate that these children had an appropriate immune balance at birth followed by no development of symptoms later in life.
This supports the idea that both an exaggerated immune response found in multitrigger wheeze and a decreased gene expression found in persistent and/or late onset wheeze results in a dysfunction contributing to the development of childhood wheeze. This indicates a limited range of healthy immune regulatory propensity already at birth.

To date, many approaches have been established in order to personalize treatment strategies in young children with wheeze and asthma and to optimize the individual treatment response. This is important when trying to avoid both overtreatment and exacerbations. The prediction of asthma development in wheezing infants has become of growing interest as there is evidence for the heterogeneity of this patient group [12] leading to remaining treatment gaps [85].

In this project, focus has been put on differences in the gene expression of pathways related to the innate immune system detected in cord blood.
These genes include the NFκB signalling pathway with its downregulation known to have a therapeutic effect on asthma [40]. It is influenced by – among others – PRRs, the inflammasome and the immunoproteasome.
Those genes were chosen for analysis as they are likely to have influence on the pathology of asthma development. TLRs are known to be potential risk genes for asthma [51], genes of the immunoproteasome and the gene encoding for Mincle – a CLR- are known to influence the T_H cell response in both human and mice [58, 65]. Genes of the NLRP3 inflammasome /IL-1R1 axis modulate airway inflammation [68] and are associated with asthma in human [71, 73] and have become of recent interest as a therapeutic target in allergic diseases [86].
The found upregulation of gene expression for said genes – especially the NLRP3/IL-1R1 axis – in children with multitrigger wheeze compared to the other phenotypes underlines the mentioned heterogeneity of asthma pathology. In this context, those children might be at higher risk for asthma development and could be filtered out for research like intervention studies.
Additionally, early viral wheeze was not associated with any significant differences in gene expression compared with healthy controls. This might indicate that those children may not benefit from an intensive treatment strategy as they are likely to 'outgrow' their symptoms. This is in line with other findings indicating that children with multitrigger wheeze benefit from a continuous use of medication whereas intermittent treatment should be applied to children with early viral wheeze [87].
Further research on the prediction of asthma development of wheezing infants could contribute to avoiding overtreatment in this subgroup.
We found differences in the expression of genes related to the pathology of asthma already at birth. However, due to the limited sample size, further research is urgently needed to confirm and better understand those findings. The on-going follow up at age 10 years of the children analyzed in this project will also add important information of future symptom development of the different phenotypes.

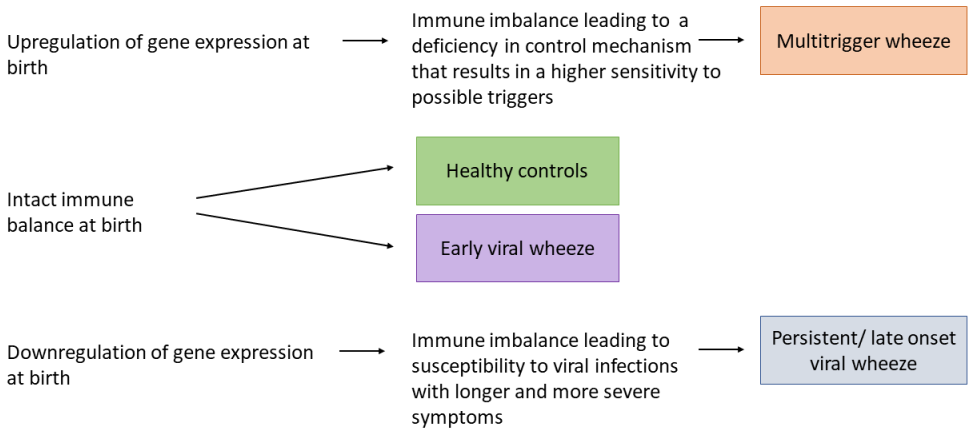


Figure 52: Visualization of the hypothesized endotype characteristics found in this project

5.1.2. Differences in gene expression considering persistency of symptoms

The more detailed phenotype analysis included the temporal pattern of wheeze symptoms leading to smaller subgroups of children within multitrigger wheeze and viral wheeze. Therefore, these findings have to be interpreted with caution. They were classified into either ‘persistent’ or ‘late onset’ viral or multitrigger wheeze, respectively.

Classification of children with multitrigger wheeze into those with persistent or late onset multitrigger wheeze unmasked the strongest upregulation in children with persistent multitrigger wheeze.
In contrast to the phenotype analysis in the four larger groups, children with persistent multitrigger wheeze did not show statistically significant differences in gene expression without immune stimulation as compared to other subgroups. However, the upregulation of the gene expression in children with persistent multitrigger wheeze after PHA stimulation was more strongly detectable even though the number of samples decreased from 14 to 4. This was detectable for all analyzed genetic pathways, including the PRRs (TLR5, TLR7, MDA-5 and Dectin1), the immunoproteasome (LMP2) and the inflammasome/IL-1R1 axis. This indicates that the immune system of children with subsequent persistent multitrigger wheeze shows an exaggerated response upon stimulation right after birth. This was also detectable after LpA stimulation for Mincle, NLRP3, Casp1 and IL-1R1.
Thus, children with persistent multitrigger wheeze may have a predisposition at birth which is visible following immune activation and may lead to uncontrolled immune regulation when exposed to triggers subsequently. This may in the long-term result in an increased risk for chronic wheeze symptoms and potentially the development of childhood asthma later in life. This is in line with results from Hallberg et al who found that early persistent wheeze was associated with the strongest lung impairment at age 16 [88]. Yet, the subgroup of children is very small, and these findings need to be replicated in larger studies. If this can be confirmed in other studies, it may be possible to identify these children already early in life and either monitor them more closely in case of symptoms or select those for potential early intervention studies.

Gene expression of children with late onset multitrigger wheeze showed some similarities to gene expression pattern of those with persistent multitrigger wheeze but differed in other points.
Similar to persistent multitrigger wheezers, children with late onset multitrigger wheeze showed an upregulation of NLRP3 and the PRRs TLR5 and TLR7 after PHA stimulation. This indicates that these phenotypes share some features in terms of increased innate immune responses. However, children with late onset multitrigger wheeze showed a downregulation after LpA stimulation for TLR7 and Mincle explaining why there was no difference detectable for the more unspecific phenotype analysis in larger groups. Additionally, this shows that the two phenotypes are different, which could be a hint that children with late onset multitrigger wheeze may better compensate the assumed immune imbalance resulting in later onset of symptoms.

Subclassification of children with viral wheeze into those with persistent and late onset viral wheeze indicated strong downregulation of gene expression in persistent viral wheezers.
Interestingly, the difference in gene expression was most strongly detectable between children with persistent viral wheeze and early viral wheeze. There was a significant downregulation in children with persistent viral wheeze for TLR7 and Mincle after LpA stimulation.
Additionally, RIG-I, LMP2 and Casp1also showed a downregulation compared to early viral wheeze in unstimulated conditions.
This might indicate a genetic background or different susceptibility for the persistency of wheezing symptoms in a viral infection. In contrast to children with early viral wheeze (symptoms only up to age 3 years), children with persistent viral wheeze (symptoms at both age 3 and age 6 years) might feature an unbalanced immune response resulting in persistency of symptoms. This may put those children at a higher risk for asthma development as they are more likely to have recurrent symptoms. Recurrence of symptoms is known to be a risk factor for asthma development and therefore is one criterion of the Asthma Predictive Index [89]. This index based upon simple clinical criteria, like parents diagnosed with asthma or evidence of sensitization, is used to determine which children under the age of 3 years are likely to develop asthma later in life[90]. In line with these findings, it has been shown previously that children with persistent wheeze are more likely to develop allergies and asthma later in life [84].

Children with late onset viral wheeze showed a trend towards increased gene expression for Mincle, LMP2 and Casp1 compared to persistent viral wheeze. These findings of differences between persistent and late onset viral wheeze, although for a small number of children, may explain less findings in the analyses of the larger, more unspecific phenotype analysis.

In summary, both children with persistent multitrigger wheeze and children with persistent viral wheeze showed an aberrant gene expression after immune system stimulation. Increased gene expression of NLRP3/IL-1R1pathways in children with persistent multitrigger wheeze might reveal a genetic risk factor for developing persistent symptoms. However, the pathophysiology of asthma development is complex with many contributing factors.
Children with persistent viral wheeze showed decreased gene expression already at birth potentially predisposing the children to an inefficient response to viral infections. This may explain why those children continuously have viral-induced wheeze later in life.
Taken together, the more detailed phenotype analysis revealed that both phenotypes with persistent symptoms showed highly differing gene expression compared to the other phenotypes. Persistency of symptoms seems to be linked with the strongest up- and downregulation of gene expression.

5.2. Confounder analysis and multiple testing

As there was a reasonable positive correlation (average correlation coefficient around 0.6; see page 36) between gene expressions, a strict multiple comparison's adjustment as Bonferroni would be overly conservative. Acknowledging the explorative character of this project and due to the restricted sample size caused by limited availability of the human blood samples we waived any correction. For that reason, there was no adjustment for multiple testing performed.

The analysis of the different phenotypes for possible confounders revealed significant differences regarding maternal asthma and maternal school education. These differences were significant for maternal asthma when comparing healthy children (0.0% maternal asthma) and children with persistent or late onset viral wheeze (22.2% maternal asthma). For maternal education, there was a statistically significant difference when comparing children with multitrigger wheeze (mean=13 years of maternal education) and children with early viral wheeze (mean= 16 years of maternal education). Therefore, a possible effect of both maternal asthma and maternal education on the gene expression was calculated by grouping the children according to the maternal asthma or maternal education status.

There was no statistically significant difference in the gene expression between the two groups for maternal asthma (maternal asthma yes/no). This finding indicates that maternal asthma has no direct influence on the gene expression of the analyzed genes resulting in no need to adjust for maternal asthma.

However, for maternal school education, three findings were significant: LMP2, LMP 7 and NLRP3 (all after PHA stimulation). Therefore, the significant findings within these genes were recalculated stratified for maternal school years. The analysis showed that the overall upregulation of gene expression in children with multitrigger wheeze remained unchanged for children with 9-13 years of maternal school education. However, for children with late onset multitrigger wheeze and 16 years of maternal education a lower gene expression of NLRP3 was indicated compared with the other phenotypes. This indicated downregulation was not statistically significant (see page 120) with p-values ranged from 0.96 and 0.78. For this reason, no general adjustment for maternal education was performed in this project. However, maternal education is known as a possible confounder for asthma development [91] even though its influence was negligible in this project.

5.3. Evaluation of methods

CBMC stimulation showed the strongest effect after LpA stimulation (see page 37). LpA is a potent stimulus of the innate immune system as its primary binding partners are monocytes, macrophages and neutrophils [92]. LpA triggers a rapid innate immune response with the release of, among others, IL-1, IL-8, leukotrienes and prostaglandins. Taken together, LpA stimulation mimics the cell signalling processes following the activation of the innate immune system [93] by bacteria.

This process is mediated – among others – by TLRs. LpA is known to potentially activate TLR4 and it has recently been shown that this process is modulated by NFκB [94].

Interestingly, most significant findings could be seen after PHA stimulation. PHA is known to stimulate T cell proliferation [95] and thereby is an activator of the adaptive immune system. However, it could be possible that PHA as a very potent stimulus might also have an indirect effect on innate immune activation. That might explain the divergence between PHA as a known T cell stimulus and finding the most effects on expression of genes related to the innate immune system after this stimulation.
Measuring gene expression in cord blood is a non-invasive method at the earliest time point available. This raises a lot of opportunities especially when it comes to finding early risk factors in order to filter out those children who would benefit from an early treatment strategy. Of course, further research is needed to define said early risk factors.
The qRT-PCR is a very specific and at the same time very sensitive method to detect even small differences in mRNA expression. Measuring cDNA levels, meaning indirect measurement of mRNA levels, cannot depict the actual translation product activity in the cell. However, it can help to find potential candidate gene and related pathways for further research.
Additionally, the measured differences in gene expression might hold the potential to be used as a predictive biomarker regardless of the actual involvement of these genes in the disease's pathogenesis.

5.4.Evaluation of the PAULINA/PAULCHEN birth cohort

The PAULINA/PAULCHEN cohort is an in depth described birth cohort with detailed information at inclusion about both the child and the parents. Additionally, it provides detailled follow-up information at both age 3 years and age 6 years with current ongoing follow-up with 10 years of age. This information offers the opportunity to further investigate the children for their consistency in the development of the defined phenotypes. The quality of patient recruitment was assured by the consistent application of inclusion and exclusion criteria. The number of children developing symptoms was in accordance with the wheeze prevalence in children that can range between 15 percent and 40 percent depending on the analyzed population [96, 97]. As the PAULINA study population was recruited based upon random selection, this led to a limited sample size.
Due to this availability of limited samples from children presenting with symptoms, the found gene expression differences between the different wheeze phenotypes need to be further investigated.
However, it is highly interesting that even with this small number of children there were significant differences between the phenotypes detectable. Nevertheless, as the number of children in the subgroups decrease, replication in larger numbers and potentially including functional studies is required.
One facet of note is that the information is based on questionnaire assessment. While some studies are critical regarding reliability [98], a number of epidemiological studies showed that questionnaire-based information was reflecting clinical phenotypes reliably [83].

5.5. Conclusion

In this project, there was a difference in the gene expressions of children with defined wheeze phenotypes detectable at birth. Showing a signature of childhood wheeze phenotypes on mRNA level, these results may contribute to finding potential new biomarkers for the prediction of asthma development following childhood wheeze. This is especially important as half of preschool children show wheezing symptoms at least once and a third of those is likely to develop asthma [97]. This highlights the necessity to filter out those children at risk in order to provide best treatment or close follow-up and at the same time to avoid overtreatment for those children that will most likely outgrow their symptoms.

In this project, insights into novel gene regulation mechanisms revealed potential new biomarkers for the prediction of childhood wheeze. As a potential new biomarker should be assessed as easily as possible in the clinic, genes with a different expression between the phenotypes under unstimulated conditions seem most promising. This has the advantage that no cell culturing is necessary in addition to the advantage of a non-invasive method of sample collection provided by cord blood. In order to distinguish between the wheeze phenotypes and to assess the personal asthma risk later in life, it seems highly interesting to further investigate the role of the inflammasome/IL-1R1 axis. In this project, the gene expression of NLRP3, Casp1 and IL-1R1 differed significantly between the wheeze phenotypes and, which is especially important, differed from the gene expression of healthy controls. Consequently, considering those genes as potential biomarkers for the prediction of childhood wheeze might be a possibility to assess the personal risk allowing a more personalized treatment strategy. However, further research is needed in order to assess the potential of these genes as predictive biomarkers of childhood wheeze.

5.6. Outlook

To further investigate the symptom development of the children and to address which actually develop to a consistent asthma phenotype or outgrow their symptoms, the ongoing follow up at age of 10 years will help to answer these questions.
Additionally, it seems highly interesting to determine which cells actually contribute to the upregulation of the gene expression. This could be further analyzed by isolating the immune cells in order to get more insight into the role of the different cell subtypes.
To assure reproducibility, it would be interesting to confirm these findings in another birth cohort.
Due to the limited sample size leading to small numbers of children especially in the subgroups considering the temporal aspect of wheeze, replication in a larger cohort with more children is necessary.

6. SUMMARY

Childhood wheeze is very common with a prevalence up to 30%, depending on study design and phenotype definition [99]. However, the clinical outcome of children wheezing within the first years of life varies widely with some children developing asthma later in life and others outgrowing their symptoms. Therefore, the necessity of grouping those children in either high-risk or low-risk for complications later in life in order to prevent both under- and overtreatment has increased over the past decades. One promising approach is endotyping childhood wheeze meaning evaluating the risk according to underlying molecular mechanisms leading to new biomarkers for the prediction of childhood wheeze.

In this project, genes related to the innate immune system and to the NFκB signalling pathway were analyzed for differences in expression on RNA level. We hypothesized that the gene expression would differ between children with multitrigger wheeze, early viral wheeze, late onset/persistent viral wheeze and healthy controls.

Genes related to the innate immune system and to the NFκB signalling pathway were chosen upon their relevance for asthma based on literature and upon preliminary experiments of our work group.
In order to measure the gene expression at the earliest time point available, cord blood mononuclear cells (CBMCs) from children of the PAULINA/PAULCHEN birth cohort [3, 74] were stimulated with either PHA or LpA and then analyzed by performing quantitative real-time PCR.
In the PAULINA/PAULCHEN birth cohort, n=283 children were recruited between 2004 and 2008 with a detailed questionnaire at birth, at age 3 years, at age 6 years and an ongoing follow up until today. Based on the questionnaires, a subsample of n=76 children were classified into healthy controls, multitrigger wheeze, early viral wheeze or late onset/persistent viral wheeze and gene expression was measured on RNA level by performing quantitative real-time PCR of cDNA.

The phenotype comparison revealed that children with multitrigger wheeze showed the highest gene expression overall and children with viral wheeze the lowest compared with the other phenotypes. This effect was most strongly detectable for genes related to the inflammasome/IL-1R1 axis (NLRP3, Casp1, IL-1R1) and remained statistically significant even when analyzing more detailed phenotypes taking into account the temporal pattern of wheeze. Even though the number of samples per group decreased, the differences were still statistically detectable indicating strong effects. Additionally, children with persistency of symptoms showed a more differing gene expression from healthy controls than those with late onset symptoms.

In this project, some candidate genes with the potential of new biomarkers for the predication of childhood wheeze were identified. Further analysis including the information of the age 10 years follow up and a more detailed understanding of the involved cell types together with the confirmation in another birth cohort is needed to

fully understand the potential of these candidate genes as new predictive biomarkers for childhood wheeze.

7. ZUSAMMENFASSUNG

Pfeifen oder Giemen bei der Ausatmung (sog. *wheeze*) in der Kindheit sind ein häufig auftretendes Symptom mit einer Prävalenz von bis zu 30% [99], je nach betrachteter Population. Jedoch unterscheidet sich das spätere klinische Bild der Kinder mit Pfeifen oder Giemen sehr deutlich: manche dieser Kinder entwickeln im späteren Leben Asthma und bei anderen Kindern verschwinden die Symptome mit zunehmendem Alter komplett. Aus diesem Grund ist die Notwendigkeit, diese Kinder nach hohem beziehungsweise niedrigem Asthmarisiko einzuteilen über die letzten Jahre enorm gestiegen, gerade im Hinblick darauf, sowohl eine Überbehandlung als auch eine medizinische Unterversorgung zu verhindern. Ein vielversprechender Ansatz für solch eine Einteilung ist die Endotypisierung dieser Kinder. Dabei wird das Asthmarisiko mit Hilfe der zugrundeliegenden molekularen Mechanismen ermittelt, was zu sogenannten Biomarkern für die Prädiktion von kindlichen Atemgeräuschen und deren weiteren Verlauf führen kann.

In dieser Arbeit wurden Gene, die mit dem angeborenen Immunsystem sowie dem NFκB Signalweg assoziiert sind, auf Unterschiede in der Expression auf RNA Level untersucht um mögliche neue Biomarker zu identifizieren. Die Hypothese lautete, dass sich die Genexpression von Kindern mit Pfeifen und Giemen (sog. *wheeze*), die durch mehrere Faktoren ausgelöst werden (*multitrigger wheeze*), solchen Kindern, die früh im Leben im Virusinfekt Atemgeräusche zeigten (*early viral wheeze*), Kindern mit Atemgeräuschen im Virusinfekt, die persistieren oder im späteren Leben auftreten (*persistent or late onset viral wheeze*), und gesunden Kontrollen voneinander unterscheiden.

Die Genauswahl erfolgte anhand der aktuellen Literatur sowie auf Grundlage von Vorarbeiten aus der Arbeitsgruppe. Um die Genexpression zu einem möglichst frühen Zeitpunkt im Leben zu messen, wurden Nabelschnurblutzellen (*CBMCs)* von Kindern aus der PAULINA/PAULCHEN Geburtskohorte [3, 74] entweder mit PHA oder LpA stimuliert und anschließend die Genexpression mit Hilfe von quantitativer real-time PCR untersucht.

Für die PAULINA/PAULCHEN Geburtskohorte wurden n=283 Kinder im Zeitraum von 2004 bis 2008 in München und Umgebung rekrutiert. Die Rekrutierung und die spätere Nachverfolgung beinhalteten einen detaillierten Fragebogen bei Einschluss, nach 3 Jahren, nach 6 Jahren und aktuell läuft die Nachbereitung nach 10 Jahren. Mit den Fragebögen als Grundlage wurde eine Untergruppe von n=76 Kindern in die verschiedenen Phänotypen (*multitrigger wheeze, early viral wheeze, persistent/late onset viral wheeze* und gesunde Kontrollen) eingeteilt und deren Genexpression auf RNA level mit Hilfe von quantitativer real-time PCR der cDNA analysiert.

Der Vergleich der unterschiedlichen Phänotypen zeigte, dass Kinder mit *multitrigger wheeze* einen generellen Anstieg der Genexpression zeigten, wohingegen Kinder mit *persistent/late onset viral wheeze* durch eine generelle Reduktion gekennzeichnet waren.

Dieser Effekt war am stärksten ausgeprägt für Gene, die mit der Inflammasom/IL-1R1 Achse assoziiert sind (NLRP3, Casp1 und IL-1R1), und blieb auch bei einer feineren Aufteilung der Phänotypen, die den zeitlichen Verlauf der Symptome berücksichtigte, erhalten. Obwohl dadurch die Anzahl der Kinder pro Gruppe sank, blieben die Unterschiede in der Genexpression nachweisbar, was auf starke Effekte schließen lässt. Zusätzlich stellte sich heraus, dass die Genexpression von Kindern mit persistierenden Beschwerden stärker von der Genexpression gesunder Kinder abwich als die von Kindern, deren Symptome erst später einsetzen.

Zusammenfassend wurden in dieser Arbeit Kandidatengene mit dem Potential eines prädiktiven Biomarkers für die Entwicklung von kindlichen pfeifenden/giemenden Atemgeräuschen identifiziert. Weitere Analysen mit dem Fokus auf die laufende 10-Jahres-Nachbereitung sowie eine vertiefende Untersuchung der beteiligten Zelltypen zusammen mit der Ergebnisbestätigung in einer anderen Geburtskohorte sind notwendig um das Potential dieser Gene als prädiktive Biomarker vollständig zu verstehen.

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9. ABBREVEATIONS

CARD	Caspase activation and recruitment domain
CASP1	Casapse 1
CBMCs	Cord Blood Mononuclear Cells
CD	Cluster of differentiation
cDNA	Complementary DNA
CI	Confidence interval
CLRs	C-type lectin receptors
C_T	Threshold cycle
DAMPs	Damage-associated molecular patterns
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
EVW	Early viral wheeze
HC	Healthy controls
IFN	Interferon
Ig	Immunoglobuline
IKK	IκBα kinase complex
IL-1R1	Interleukin 1 receptor, type I
IQR	Inter quartile range
LMP2	Low molecular mass polypetide 5
LMP7	Low molecular mass polypetide 7
LOM	Late onset multitrigger wheeze
LOVW	Late onset viral wheeze
LpA	Lipid A
M	Media (unstimulated)
MDA-5	Melanoma differentiation associated gene 5
MHC	Major histocompability complex
mRNA	Messenger RNA
MT	Multitrigger wheeze
NFκB	Nuclear factor `kappa-light-chain enhancer´ of activated B-cells
NLRP3	NOD-like receptor family, pyrin domain containing 3
NTC	Non-template control
PAMPs	Pathogen-associated molecular pattern
PBMCs	Peripheral Blood Mononuclear Cells
PHA	Phytohaemagglutinin
PMT	Persistent multitrigger wheeze
PVW	Persistent viral wheeze
PPRs	Pattern recognition receptors
qRT PCR	Quantitative real-time polymerase chain reaction
RIG-I	Retinoic acid-inducible gene I
RLRs	RIG-I like receptors
RNA	Ribonucleic acid
T_H	T-helper cell
TNF	Tumor necrosis factor
TLR	Toll-like receptor
T_{reg}	Regulatory T-cells
VW	Viral wheeze

10. TABLE INDEX

11. FIGURE INDEX

12. ATTACHMENTS

12.1. Declaration of consent for PAULINA and PAULCHEN

EINVERSTÄNDNIS

Zur Nabelschnurblutstudie PAULINA

„Das Immunsystem des Neugeborenen: Charakterisierung des Phänotyps und Funktion von Nabelschnurblut im Rahmen von Endotoxinstimulation“

Vor und Nachname der Mutter: .....

Name des Kindes: .....

Anschrift: .....

.....

Telefon: .....

Hiermit erkläre ich/wir mein/unser Einverständnis, an der Studie teilzunehmen. Ich/Wir wurde/n über das Projekt und die Risiken der Teilnahme informiert. Ich/wir bin/sind damit einverstanden, dass bei der Mutter bei der Routineblutabnahme Blut für eine Allergietestung und aus dem Nabelschnurblut nach Entbindung ca. 20-30 ml Blut entnommen werden. Zudem sind wir einverstanden, dass für evtl. spätere Untersuchungen DNA von Mutter und Nabelschnurblut eingefroren wird.

Ich/Wir kann/können diese Einverständniserklärung jederzeit ohne jegliche Folgen widerrufen.

Das Informationsblatt habe ich/wir gelesen und ich/wir hatte/n ausreichend Zeit, diese Entscheidung zu überlegen. Alle meine/unsere Fragen wurden beantwortet. Eine Kopie des Informationsblattes und der Einverständniserklärung habe ich/wir erhalten.

....

Ort, Datum Unterschift der Mutter

EINVERSTÄNDNIS

Zur Nabelschnurblutstudie PAULCHEN

„Das Immunsystem des Neugeborenen: Charakterisierung des Phänotyps und Funktion von Nabelschnurblut im Rahmen von Endotoxinstimulation“

Vor und Nachname der Mutter: .....

Name des Kindes: .....

Anschrift: .....

.....

Telefon: .....

Hiermit erkläre ich/wir mein/unser Einverständnis, an der Studie teilzunehmen. Ich/Wir wurde/n über das Projekt und die Risiken der Teilnahme informiert. Ich/wir bin/sind damit einverstanden, dass bei der Mutter bei der Routineblutabnahme Blut für eine Allergietestung und aus dem Nabelschnurblut nach Entbindung ca. 20-30 ml Blut entnommen werden. Zudem sind wir einverstanden, dass für evtl. spätere Untersuchungen DNA von Mutter und Nabelschnurblut eingefroren wird.

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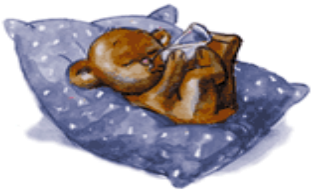
....

Ort, Datum Unterschrift der Mutter

12.2. Questionnaire for age six years follow-up for PAULINA and PAULCHEN

For both, PAULINA and PAULCHEN age six years follow-up, similar questionnaires were used. The only differences in the PAULCHEN questionnaire affected questions regarding the home and life situations. For this reason, only the PAULINA age 6 years questionnaire is shown below.



PAULINA

Fragebogen zum **6. Lebensjahr**

Ihres Kindes

Datum:_____ Studiennummer:_____

Fragebogen für die Eltern

Wir freuen uns, dass Sie bereit sind weiterhin an der Paulina Studie teilzunehmen. Bitte kreuzen Sie die folgenden Fragen an. Ihre Antworten werden vertraulich behandelt. Wenn Sie eine Frage nicht beantworten möchten, lassen Sie sie bitte aus.

Wir danken Ihnen herzlich für Ihre Mitarbeit!

Wir beginnen mit Fragen zu pfeifenden und keuchenden Atemgeräuschen. Mit pfeifenden Atemgeräuschen meinen wir ein pfeifendes Geräusch, das aus dem Brustkorb kommt, aber nicht geräuschvolles Atmen durch die Nase.

Hat Ihr Kind <u>jemals pfeifende bzw. keuchende Atemgeräusche</u> gehabt?

Ja ☐

Falls Ja,

wann sind diese zum ersten Mal aufgetreten:

Nein... ☐ ⇒ weiter mit Frage 12

Hatte Ihr Kind <u>in den letzten 3 Jahren</u> pfeifende bzw. keuchende Atemgeräusche?

Ja ☐

Nein ☐ ⇒ weiter mit Frage 12

Wie oft hatte Ihr Kind <u>in den letzten 12 Monaten</u> pfeifende bzw. keuchende Atemgeräusche?

Gar nicht ☐

1-3 mal ☐

4-12mal ☐

Mehr als 12 mal ☐

Hatte Ihr Kind <u>in den letzten 12 Monaten</u> jemals <u>Atemnot</u>, als die pfeifenden/ keuchenden Atemgeräusche auftraten?

Ja ☐

Nein ☐

Wie häufig ist Ihr Kind <u>in den letzten 12 Monaten nachts</u> wegen pfeifender oder keuchender Atemgeräusche aufgewacht?

Seltener als einmal pro Monat ☐

Einmal pro Monat ☐

Mindestens zweimal pro Monat ☐

Wodurch wurden bei Ihrem Kind die <u>pfeifenden / keuchenden Atemgeräusche</u> ausgelöst?

	Ja	**Nein**
Anstrengung	☐	☐
Erkältung	☐	☐
Kontakt mit Tieren	☐	☐
Kontakt mit Hausstaub	☐	☐
Kontakt mit Gras	☐	☐
Sonstiges	☐	☐

_____

Wie häufig hatte Ihr Kind <u>in den letzten 12 Monaten</u> pfeifende oder keuchende Atemgeräusche, <u>ohne dass es erkältet war</u>?

Nie ☐

Seltener als einmal pro Monat ☐

Einmal pro Monat ☐

Mindestens zweimal pro Monat ☐

Ist das Kind zwischen diesen Episoden völlig beschwerdefrei?

Ja ☐ ⇒ weiter mit Frage 12

Nein ☐

Hat Ihr Kind zwischen diesen Episoden folgende Beschwerden

bei Anstrengung?

	Ja	**Nein**
Husten	☐	☐

Pfeifende Atemgeräusche ☐ ☐

Atemnot ☐ ☐

Sonstiges:_____

Bei Temperaturwechsel/Nebel?

	Ja	**Nein**
Husten	☐	☐
Pfeifende Atemgeräusche	☐	☐
Atemnot	☐	☐

Sonstiges:_____

Nachts?

	Ja	**Nein**
Husten	☐	☐
Pfeifende Atemgeräusche	☐	☐
Atemnot	☐	☐

Sonstiges:_____

Sonstige Beschwerden?

_____

Hat Ihr Kind jemals <u>in den letzten 3 Jahren</u> von einem Arzt Medikamente gegen pfeifende oder keuchende Atemgeräusche, oder Giemen oder Atemnot verschrieben bekommen?

(Gemeint sind damit nicht nur Medikamente zum Schlucken, sondern auch Inhalationen oder Sprays)

Ja ☐

Nein ☐ ⇒ weiter mit Frage 12

Welche Medikamente waren dies?

Bitte geben Sie den Markennamen möglichst genau an! Und sofern Sie es wissen die Dosis sowie den Zeitraum, in dem das Medikament eingenommen wurde.

_____

_____

Wurde bei Ihrem Kind jemals von einem Arzt ein Allergietest durchgeführt?

	Ja	**Nein**
Ein Hauttest	☐	☐
Ein Bluttest	☐	☐
Ein anderer Test, z.B. Bioresonanz	☐	☐

Welche Allergie wurde dabei festgestellt?

	Ja	**Nein**
Gegen Pollen	☐	☐
Gegen Hausstaub(milben)	☐	☐
Gegen Tiere	☐	☐
Gegen Nahrungsmittel	☐	☐
Andere:_____	☐	☐

Hat Ihr Kind jemals <u>in den letzten 3 Jahren</u> von einem Arzt Medikamente aus einem anderen Grund verschrieben bekommen?

(Gemeint sind damit nicht nur Medikamente zum Schlucken, sondern auch Inhalationen oder Sprays)

Ja ☐

Nein ☐ ⇒ weiter mit Frage 16

Welche Medikamente waren dies?

Bitte geben Sie den Markennamen möglichst genau an! Und sofern Sie es wissen die Dosis sowie den Zeitraum in dem das Medikament eingenommen wurde.

_____

_____

_____

Es folgen Fragen zu Beschwerden der Nase und der Augen

Hat Ihr Kind <u>jemals</u> Niesanfälle oder eine laufende, verstopfte oder juckende Nase, obwohl es <u>nicht</u> erkältet war?

Ja ☐

Falls Ja, wann ist dies zum ersten Mal aufgetreten:

Nein ☐ ⇒ weiter mit Frage 21

Hatte Ihr Kind <u>in den letzten 3 Jahren</u> Niesanfälle oder eine laufende, verstopfte oder juckende Nase, obwohl es <u>nicht</u> erkältet war?

Ja ☐

Nein ☐ ⇒ weiter mit Frage 21

Hatte Ihr Kind <u>in den letzen 12 Monaten</u> gleichzeitig mit diesen Nasenbeschwerden juckende oder tränende Augen?

Ja ☐

Nein ☐

Wann <u>in den letzen 12 Monaten</u> traten diese Nasen-Beschwerden auf?

Mehrere Antworten sind möglich.

Januar ☐ Mai ☐ September ☐

Februar ☐ Juni ☐ Oktober ☐

März ☐ Juli ☐ November ☐

April ☐ August ☐ Dezember ☐

Ist von einem Arzt bei Ihrem Kind schon einmal Heuschnupfen oder eine allergische Rhinitis bzw. Rhinokonjunktivitis festgestellt worden?

Ja ☐

Nein ☐

Es folgen Fragen zu Hauterkrankungen

Hatte Ihr Kind jemals eine Neurodermitis/atopische Dermatitis/ atopisches Ekzem

Ja ☐

Falls Ja, wann ist diese zum ersten Mal aufgetreten: _____

Nein ☐ ⇒ weiter mit Frage 31

Wurde bei Ihrem Kind die Diagnose einer Neurodermitis/atopischen Dermatitis/ atopisches Ekzem von einem Arzt gestellt?

Ja ☐

Nein ☐

Hatte Ihr Kind <u>in den letzten 3 Jahren</u> eine Neurodermitis/atopische Dermatitis/ atopisches Ekzem

Ja ☐

Nein ☐

War der Hautausschlag je an einer der folgenden Stellen?

	Ja	Nein
Gesicht	☐	☐
Hals	☐	☐
Ellenbeugen / Kniekehlen	☐	☐
Hand- / Fußgelenke	☐	☐
Brust/Rücken	☐	☐

Hat sich die Lokalisation des Ausschlages im Laufe der Zeit geändert?

Ja…….☐ Nein…….☐

Falls Ja, wo war er zu Beginn? Wo befindet er sich heute?

Zu Beginn:

	Ja	**Nein**
Gesicht	☐	☐
Hals	☐	☐
Ellenbeugen / Kniekehlen	☐	☐
Hand- / Fußgelenke	☐	☐
Brust/Rücken	☐	☐

Heute:

	Ja	Nein
Gesicht	☐	☐
Hals	☐	☐
Ellenbeugen / Kniekehlen	☐	☐
Hand- / Fußgelenke	☐	☐
Brust/Rücken	☐	☐

Wenn Sie die Zeiten, in denen Ihr Kind diesen Hautausschlag hatte, zusammenzählen: Wie lange haben Sie diesen Hautausschlag insgesamt beobachtet?

Für insgesamt weniger als 3 Monate ☐

Für insgesamt 3-6 Monate ☐

Für insgesamt 6-12 Monate ☐

Für länger als 12 Monate ☐

Ist der Hautausschlag wieder völlig verschwunden, oder „kommt und geht" der Hautausschlag?

Der Hautausschlag ist vollständig

Verschwunden ☐

Der Hautausschlag „kommt und geht" ☐

Der Hautausschlag ist noch da ☐

Wie alt war Ihr Kind, als der Hautausschlag vollständig verschwunden ist?

_____ Monate

Wie häufig ist Ihr Kind <u>nachts</u> wegen Juckreiz aufgewacht?

Seltener als einmal pro Monat oder nie ☐

Einmal pro Monat ☐

Mindestens zweimal pro Monat ☐

Haben Sie die Haut Ihres Kindes <u>in den letzten 12 Monaten</u> mit einer cortisonhaltigen Creme / Salbe oder einer Tacrolimus- bzw. Pimecrolimus-haltigen Salbe (Protopic, Elidel) behandelt?

Ja ☐

Nein ☐

Es folgen Fragen zu Nahrungsunverträglichkeiten oder –allergien

Hat Ihr Kind eine Nahrungsmittelallergie?

Ja ☐

Nein ☐ ⇒ weiter mit Frage 34

32. Wie äußert sich diese Nahrungsmittelallergie?

Ausschlag/rote Flecken um den Mund herum ☐

Ausschlag/rote Flecken an anderen Körperstellen ☐

Schwellung der Lippen ☐

Juckreiz ☐

Durchfall ☐

Erbrechen ☐

Verschlechterung der Neurodermitis ☐

Pfeifende Atemgeräusche ☐

Atemnot ☐

Kreislaufreaktion/Blutdruckabfall ☐

Sonstiges:

33. Auf welche Nahrungsmittel reagiert Ihr Kind?

	Ja	Nein
Milch und Milchprodukte	☐	☐
Hühnereier	☐	☐
Fisch	☐	☐
Weizenmehl oder andere Getreideprodukte	☐	☐
Nüsse	☐	☐
Soja	☐	☐
Zitrusfrüchte	☐	☐
Anderes Obst oder Gemüse	☐	☐

Andere Nahrungsmittel ☐ ☐

Welche? _____

Es folgen Fragen zu anderen Erkrankungen

34. Wurde bei Ihrem Kind jemals <u>von einem Arzt/einer Ärztin</u> eine spastische Bronchitis, obstruktive Bronchitis oder asthmatische Bronchitis diagnostiziert?

Nein, nie ☐

Ja, einmal ☐

Ja, mehrmals ☐

35. Wurde bei Ihrem Kind in den letzten 12 Monaten <u>von einem Arzt/einer Ärztin</u> eine der folgenden Diagnosen gestellt?

	Ja	**Nein**
Asthma	☐	☐
Neurodermitis, atopische Dermatitis	☐	☐
oder endogenes Ekzem	☐	☐
Allergische Rhinitis/Heuschnupfen	☐	☐

36. Hatte Ihr Kind bisher eine der folgenden Erkrankungen <u>nach dem dritten Lebensjahr</u>?

	Ja	Nein
Mittelohrentzündung	☐	☐
Pseudokrupp	☐	☐
Lungenentzündung	☐	☐
Bronchitis	☐	☐
Bronchiolitis	☐	☐
Keuchhusten	☐	☐
Andere Infektionen	☐	☐

Welche? _____

Waren stationäre Aufenthalte im Krankenhaus notwendig ☐ ☐

Warum? _____

Angaben zur Wohnungs- und Lebenssituation

37. **A) Wie viele <u>jüngere</u> Geschwister hat Ihr Kind?**

Bitte auch Stiefgeschwister mitzählen, die in Ihrer Familie leben!

Schwestern..... Brüder.....

B) Wie viele <u>ältere</u> Geschwister hat Ihr Kind?

Bitte auch Stiefgeschwister mitzählen, die in Ihrer Familie leben!

Schwestern.....Brüder.....

38. **Bitte notieren Sie Name und Geburtsdatum der Geschwister Ihres Kindes. Bitte auch Stiefgeschwister mitzählen, die in Ihrer Familie leben!**

Name	Mädchen	Junge	Geburtsdatum
_____	☐	☐	___/___/_____
_____	☐	☐	___/___/_____
_____	☐	☐	___/___/_____

39. **Wird Ihr Kind <u>regelmäßig</u> zusammen mit anderen Kindern durch eine Tagesmutter oder bei den Großeltern betreut? Die eigenen Geschwister sind dabei nicht gemeint.**

Ja, ☐

Mit wie vielen anderen Kindern: _____

Nein ☐

40. **Wird Ihr Kind <u>regelmäßig</u> zusammen mit anderen Kindern in einer Kinderkrippe oder im Kindergarten betreut? Die eigenen Geschwister sind dabei nicht gemeint.**

Ja, ☐

Mit wie vielen anderen Kindern? _____

Nein ☐

41. **Welche der folgenden Haustiere haben/hatten Sie innerhalb der Wohnung?** ***Mehrere Antworten sind möglich.***

Keine ☐

Hund ☐

Katze ☐

Hamster ☐

Meerschweinchen ☐

Kaninchen ☐

Vögel ☐

Aquarium (Fische) ☐

Sonstige ☐

Welche: _____

A) Darf oder durfte sich eine Katze <u>im Zimmer</u>, in dem Ihr Kind schläft aufhalten?

Ja ☐

Nein ☐

B) Darf oder durfte sich eine Katze <u>im Bett</u> Ihres Kindes aufhalten?

Ja ☐

Nein ☐

C) Darf oder durfte sich ein Hund <u>im Zimmer</u>, in dem Ihr Kind schläft aufhalten?

Ja ☐

Nein ☐

D) Darf oder durfte sich ein Hund <u>im Bett</u> Ihres Kindes aufhalten?

Ja ☐

Nein ☐

42. **Hat Ihr Kind sonst regelmäßig (ca. 1x/Woche) Kontakt zu Tieren (z.B. in der Wohnung von Freunden/ Verwandten)?** ***Mehrere Antworten sind möglich.***

	Ja	Nein
Hund	☐	☐
Katze	☐	☐
Sonstige	☐	☐

Welche:_____

43. **Gibt es in Ihrer Wohnung Feuchtigkeitsflecken bzw. Schimmelbefall an Wänden oder Decken?**

Feuchtigkeitsflecken in Bad oder Küche sind dabei nicht gemeint, sondern nur in Räumen wie Wohnzimmer, Schlafzimmer oder Kinderzimmer.

	Ja	**Nein**
Feuchtigkeitsflecken, aber ohne Schimmelbefall	☐	☐

	Ja	Nein
Feuchtigkeitsflecken mit Schimmelbefall	☐	☐

Es folgen Fragen zum Rauchverhalten

44. **Rauchen Sie oder Ihre Familie in Ihrer Wohnung/Haus?**

Ja ☐

Nein ☐

45. **Haben Sie und Ihre Familie <u>in den letzten 12 Monaten</u> mit dem Rauchen in der Wohnung aufgehört bzw. das Rauchen innerhalb der Wohnräume eingeschränkt?**

Ja ☐

Nein ☐

Es wurde nie geraucht ☐

46. **Wie viele Zigaretten werden durchschnittlich am Tag <u>in Ihrer Wohnung</u> (damit meinen wir auch die Küche) geraucht? Zigaretten, die auf dem Balkon oder der**

Terrasse geraucht werden, brauchen nicht mitgezählt zu werden. Wie viele davon von... (keine=0)

Mutter _____ pro Tag

Partner_____ pro Tag

Andere Personen _____ pro Tag

Insgesamt _____ pro Tag

Haben Sie noch weitere Kommentare zum Fragebogen oder allgemein?

_____
_____
_____
_____
_____
_____
_____

Wir danken Ihnen herzlich für das

Ausfüllen des Fragebogens!

Bei Fragen können Sie sich jederzeit gerne an uns wenden.

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12.3. Stratification for maternal school years

As maternal education was revealed as a possible confounder (see Table 9), gene correlation of gene expression and maternal school years was analyzed. For LMP2, LMP7 and NLRP3, there were significant findings after PHA stimulation. In a second step, the found findings within these genes were analyzed stratified for maternal school years.

Table 50: analysis of affected findings stratified for maternal school years

	Trends and significant findings	Stratified for school years years	
LMP2 after PHA stimulation	MT > HC p-value= 0.056	9	---
		10	MT > HC p-value= 0.19
		13	MT > HC p-value= 0.095
		16	MT < HC p-value= 0.8
LMP7 after PHA stimulation	MT > EVW p-value= 0.084	9	---
		10	MT > EVW p-value= 0.4
		13	MT > EVW p-value= 0.33
		16	MT < EVW p-value= 0.97
NLRP3 after PHA stimulation	MT > VW p-value= 0.005 MT > EVW p-value= 0.013 MT > HC p-value= 0.015	9	MT > VW p-value= 0.67 --- ---
		10	MT > VW p-value= 0.4 MT > EVW p-value = 1.0 MT > HC p-value = 0.19
		13	MT > VW p-value= 0.33 MT > EVW p-value = 0.67 MT > HC p-value = 0.57
		16	MT > VW p-value= 0.17 MT > EVW p-value = 0.31 MT > HC p-value = 0.37

Table 51: analysis of affected findings in the more detailed phenotype subgroups stratified for maternal school years

	Trends and significant findings	Stratified for school years years	
LMP2 after PHA stimulation	PMT > HC p-value= 0.0016 PMT > EVW p-value= 0.033 PMT > LOVW p-value= 0.063	9	---
		10	PMT > HC p-value= 0.19 PMT < EVW p-value= 1.0
		13	---
		16	PMT > HC p-value= 0.12 PMT > EVW p-value= 0.08 PMT > LOVW p-value= 0.27
NLRP3 after PHA stimulation	PMT> HC p-value= 0.052 PMT> EVW p-value= 0.024 PMT> PVW p-value= 0.058 PMT> LOVW p-value= 0.057	9	---
		10	PMT > HC p-value= 0.57 PMT < EVW p-value= 0.67 ---
		13	----
		16	PMT > HC p-value= 0.052 PMT > EVW p-value= 0.017 PMT > PVW p-value= 0.19 PMT > LOVW p-value= 0.13
NLRP3 after PHA stimulation	LOM > HC p-value= 0.063 LOM > EVW p-value= 0.066 LOM > PVW p-value= 0.072 LOM > LOVW p-value= 0.036	9	---
		10	LOM > HC p-value= 0.19 LOM > EVW p-value= 0.67 LOM > PVW p-value= 0.67 LOM > LOVW p-value= 0.67
		13	LOM > HC p-value= 0.57 LOM > EVW p-value= 0.67 LOM > PVW p-value= 0.33 LOM > LOVW p-value= 0.67
		16	LOM < HC p-value= 0.96 LOM < EVW p-value= 0.95 LOM < PVW p-value= 0.78 LOM < LOVW p-value= 0.96

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14. LEBENSLAUF