Acute wheeze-specific gene module shows correlation with vitamin D and asthma medication

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Gene expression profiles at acute preschool wheeze correlate with vitamin D, and asthma medication and lung function several years later. These profiles provide candidate prognostic biomarkers and support the role of immune response in preschool wheeze. <http://bit.ly/318Ilde>

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ABSTRACT

Background: Airway obstruction and wheezing in preschool children with recurrent viral infections are a major clinical problem, and are recognised as a risk factor for the development of chronic asthma. We aimed to analyse whether gene expression profiling provides evidence for pathways that delineate distinct groups of children with wheeze, and in combination with clinical information could contribute to diagnosis and prognosis of disease development.

Methods: We analysed leukocyte transcriptomes from preschool children (6 months–3 years) at acute wheeze (n=107), and at a revisit 2–3 months later, comparing them to age-matched healthy controls (n=66). RNA-sequencing applying GlobinLock was used. The cases were followed clinically until age 7 years. Differential expression tests, weighted correlation network analysis and logistic regression were applied and correlations to 76 clinical traits evaluated.

Findings: Significant enrichment of genes involved in the innate immune responses was observed in children with wheeze. We identified a unique acute wheeze-specific gene-module, which was associated with vitamin D levels ($p<0.005$) in infancy, and asthma medication and FEV₁%/FVC (forced expiratory volume in 1 s/forced vital capacity) ratio several years later, at age 7 years ($p<0.005$). A model that predicts leukotriene receptor antagonist medication at 7 years of age with high accuracy was developed (area under the curve 0.815, 95% CI 0.668–0.962).

Interpretation: Gene expression profiles in blood from preschool wheezers predict asthma symptoms at school age, and therefore serve as biomarkers. The acute wheeze-specific gene module suggests that molecular phenotyping in combination with clinical information already at an early episode of wheeze may help to distinguish children who will outgrow their wheeze from those who will develop chronic asthma.

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Introduction

Recurrent viral wheeze in preschool children is a major clinical problem that requires high healthcare and economical resources. In the majority of infants, wheeze is a transient condition, but wheeze caused by early rhinovirus infections has been associated with a significantly increased risk of asthma later in childhood [1]. In a recent study, risk of asthma development was associated with the number of respiratory episodes in the first years of life, but not with specific viruses or bacteria [2]. Thus, whether infection causes asthma or serves as a marker for genetically predisposed individuals is yet unknown. Factors that determine the persistence or remittance of preschool wheeze remain unclear, but exposure to tobacco smoke is a confirmed risk factor [3], and other factors, *e.g.* sensitisation and viral infections in early life are possible risk factors [4]. Early identification of preschool children at risk of developing chronic asthma would make it possible to provide more specific therapeutic interventions, which might even interfere with the disease trajectory, and help to improve the prognosis.

Today, no simple tools or biomarkers are available to predict the long-term outcome of preschool children with wheeze. Interferons (IFNs) were originally identified through their ability to contribute to the viral resistance of cells, but their role is much broader, with involvement in both the innate and adaptive immune response [5, 6]. Three types of IFNs are described: type I (*e.g.* IFN- α , - β), type II (IFN- γ) and type III (IFN- λ), which all use distinctive, but related, multimeric receptors [7]. During a respiratory viral infection, an increase of IFN-I and -III occurs, followed by induction of cytokines and accumulation of immune cells [8, 9]. Significant alteration of serum IFN levels has been found in relation to asthma exacerbation upon rhinovirus infection [10].

Key molecular findings have been made for asthma with global gene expression studies in leukocytes, bronchial biopsies and epithelial cells [11]. However, longitudinal studies including gene expression profiles in preschool children with wheeze are rare. In an attempt to identify gene expression profiles that could be used for prognosis of asthma development and increase the understanding of the pathophysiology, we have analysed gene expression profiles in acute wheeze in relation to clinical characteristics in a longitudinal cohort of preschool children with wheeze.

Materials and methods

A full description is provided in the supplementary material.

Ethics

The study protocol was approved by the regional ethics committee of Karolinska Institutet, Stockholm (Dnr 2008/378-31/4 and 2014/399-31/3). Written informed consent was obtained from parents and/or legal guardians of all children.

Study design and subject enrolment

The children in this study are part of a longitudinal study on preschool children ≥ 6 months to 3 years old with wheezing, enrolled between 2008 and 2012, recruited consecutively when visiting the paediatric emergency department at Astrid Lindgren Children's Hospital (Stockholm, Sweden) because of acute wheezing [12, 13]. Diagnosis of acute wheeze was based on a clinical diagnosis made by the treating physician at the paediatric emergency department. The enrolment criteria were confirmed by the study doctor. Of children with acute wheeze, 80% were hospitalised for ≥ 24 h [12]. The children came to a revisit 2–3 months later (median 12 weeks), and thereafter annually to the same paediatrician and allergologist (study doctor KSH) until they reached school age. The children are well characterised with clinical examinations, standardised questionnaires and biological sampling at all visits. Families documented some characteristics, *e.g.* medication, contact with healthcare, days of absence due to illness during the year preceding each visit. Lung function tests at age 7 years were performed, as well as the Childhood Asthma Control Test (C-ACT). Included in this study are the acute visit (transcriptomics and clinical information), the first revisit after 2–4 months (transcriptomics and clinical information), and the annual visit at 7 years of age (clinical information). For inclusion and exclusion criteria see table 1. During the same recruitment period, age-matched healthy control children were recruited (table 1). For detailed information of the study design see figure 1, and clinical parameters see supplementary table E1. In total, 334 samples were included (figure 2b). For 80 children, transcriptome profiles were available from both acute wheeze and from the revisit.

Definitions of clinical parameters

For further details, see supplementary table E1. Acute wheeze was based on a clinical diagnosis made by the treating physician at the paediatric emergency department [12]. At the visit at 7 years, the children were examined by the study doctor. Asthma at age 7 years (7Y_ASTHMA_GA2LEN) was defined as a positive answer to either "Have you had an attack of asthma in the past 12 months?" or "Are you currently

TABLE 1 Inclusion and exclusion criteria

	Inclusion criteria	Exclusion criteria
Children with wheeze/asthma	Age 6–48 months Presenting at the emergency department with acute symptoms of wheeze	Prematurity (birth before 36 gestational weeks) Any chronic disease Any simultaneous complications such as sepsis, bacterial pneumonia, diabetes at the time point of inclusion
Healthy children	Age 6–48 months Otherwise healthy	Prematurity (birth before 36 gestational weeks) A history of bronchial obstruction/asthma [#] Known sensitisation to airborne allergens Any chronic disease

[#]: this criterion is aimed at excluding wheezing children at enrolment.

taking or have you during the past 12 months taken any medication for asthma, including short-acting β_2 -agonists, inhaled corticosteroids and montelukast?”, modified from [14]. In addition, allergic asthma (7Y_ASTHMA_ALLERGIC) was defined as asthma with allergic sensitisation and clinical symptoms of allergy until the age of 7 years. (7Y_FEV₁%_FVC_RATIO) refers to the ratio between forced expiratory volume in 1 s (FEV₁) and forced vital capacity (FVC) at the visit at 7 years. (7Y_LTRA) refers to leukotriene receptor antagonist medication during the year preceding the visit at 7 years. Self-reported

FIGURE 1 Overview of the study design. Children were recruited consecutively when visiting the paediatric emergency department because of acute wheezing. Diagnosis of acute wheeze was based on a clinical diagnosis made by the treating physician at the paediatric emergency department. The enrolment criteria were confirmed by the study doctor [12]. The children came back for a revisit 2–3 months later (median 12 weeks). At the visit at 7 years, the children were examined by the study doctor. Age-matched healthy control children were recruited during the same time period. Guardians and children responded to questions in structured interviews concerning medication, contact with healthcare, days of absence due to illness during the year preceding each visit, and also reported symptoms of allergy and eczema. Asthma at 7 years of age was defined as a positive answer to either “Have you had an attack of asthma in the past 12 months?” or “Are you currently taking or have you during the past 12 months taken any medication for asthma, including short-acting β_2 -agonists, inhaled corticosteroids and montelukast?”, modified from [14]. Allergic asthma at the age of 7 years was based on atopy in blood samples at the first revisit and clinical history of allergy until the age of 7 years. Numbers of individuals included in the final analyses are shown. See also the supplementary material and methods and supplementary table E1. RV: rhinovirus; C-ACT: Childhood Asthma Control Test.

FIGURE 2 Overview of the transcriptome analysis. a) Number of samples available (n=334) and included in the study after sequencing quality control (n=267), and differentially expressed genes (DEGs) between the groups and genes categorised into modules by their expression profiles using weighted correlation network analysis (WGCNA), according to the analysis steps. b) Similarity of the expression profiles for the different sample groups by principal component (PC) analysis. Sample group is annotated. c) Similarity of the expression profile for the different groups by hierarchical clustering. Sample group, cell counts of neutrophils and lymphocytes (10^6 cells· μL^{-1}) are annotated. Both b) and c) illustrate the 267 samples on the leukocyte transcriptome profile of the 1476 protein coding genes, which showed significant variation after normalisation fluctuated significantly. CTRL: healthy controls; ACW: acute wheeze; REV: revisit.

asthma control test at the visit at 7 years (7Y_ASTHMA_CONTROL_TEST) was assessed using the C-ACT [15].

Sampling and RNA extraction

Blood samples were collected both at the acute visit and the revisit for the wheezing children, and at recruitment for the healthy controls. The legal guardian filled out a standardised questionnaire, as detailed previously [12, 13]. Total RNA was extracted from buffy coat, and a RNA integrity number >8 was used as RNA quality cut-off for inclusion.

RNA sequencing and statistical analyses

A transcriptome sequencing (RNA-seq) method that targets the 5'-ends of RNA transcripts, known as STRT [16], was applied [17]. In brief, 80 ng of total RNA from each individual was used for the library preparation. To suppress uninformative globin gene transcripts originating from red blood cells, RNA samples were treated with GlobinLock oligonucleotides [17]. The samples were subdivided into eight 48-plex libraries. Each library was sequenced on three HiSeq2000 flow-cell lanes (Illumina, San Diego, CA, USA) using Illumina TruSeq v3 chemistry. The raw sequences were processed, aligned and summarised using the STRTprep pipeline v3 [16]. Library bias was corrected by an approximation-based approach [18], and spike-in based normalisation was applied. The level of significance of variation between samples was evaluated by comparison with the technical variation of spike-in RNAs [16]. Outlier samples were excluded (supplementary figure E1). Significant differential expression between the sample groups was tested using SAMstr [19]. In the differentially expressed gene analyses, false discovery rates were estimated by permutation, as described in Li and Tibshirani [20]. Hierarchical clustering was performed using the Spearman correlation distance and Ward clustering methods. Gene set enrichment analysis was performed using EnrichR [21]. WGCNA was applied according to the developers' recommendations [22].

Results

When analysing the transcriptome of peripheral blood leukocytes, the acute wheeze samples differed the most from the others. A unique acute wheeze-specific gene module, associated with vitamin D levels in infancy, and asthma medication and FEV₁/FVC ratio several years later, at age 7 years, was identified. This gene module was shown to be a predictor of LTRA medication at 7 years with high accuracy. Another gene module showed association to allergic asthma at 7 years.

Transcriptome profiling

In total, 334 preschool children were included in the current study. 67 samples were excluded for technical reasons, failing quality control (figure 2a, supplementary figure E1). 267 samples were included in the statistical analyses (figure 2a, table 2). Unsupervised clustering of the samples according to the expression profile suggested clear differences between acute wheeze and controls, while cases at revisit were more similar to controls (figure 2b and c). Significantly differentially expressed genes were identified between the groups (figure 2a, supplementary table E2) and co-regulated genes in each group were classified into modules using WGCNA implemented in R, as described later (figure 2a, supplementary table E3).

Differentially expressed genes in preschool children with wheeze in acute phase and at revisit

In order to characterise the expression profile of acute wheeze, we compared the expression of the acute wheeze samples to the revisit and the control samples. The majority (96%, 391 out of 407) of the genes upregulated in acute wheeze when compared to controls were also upregulated when compared to revisit samples in the 80 sample pairs available from both visits. This was expected, given the similarity between the expression profiles of the revisit and control samples (figure 2b and c). Gene ontology analyses of the 391 consistently upregulated genes revealed biological functions with plausible relevance to wheeze, such as neutrophil activation, cytokines and interferon signalling pathways, as well as inflammatory response and negative regulation of viral replication (figure 3a). The increased levels of neutrophils in the group of children with acute wheeze are in agreement with enrichment of genes involved in neutrophilic activity (figures 2c and 3a). Gene enrichment analyses showed highly overlapping genes in the categories IFN-signalling pathways and negative regulation of viral replication, supporting the importance of viral infections in preschool wheeze. The majority (90%, 371 out of 412) of the downregulated genes were consistent between the comparisons, and gene ontology analyses revealed mainly biological functions related to defence against viral infection (figure 3b).

The 170 upregulated genes specific for the paired samples between acute wheeze and revisit showed neutrophilic involvement, and mast cell activation and degranulation, as well as cytokine and IFN-I-mediated signalling pathways (data not shown). This probably reflects phenotype-relevant biological

TABLE 2 Basic characteristics of the children

	Wheeze acute visit	Wheeze revisit	Healthy controls
Subjects	107*	94	66
Male	68 [64]	48 [51]	52 [82]
Age months median (range)	17 [7–42]	19 [9–45]	17 [6–44]
Birth weight kg	3.4 [3.0–3.7]*	3.4 [3.1–3.8]	3.5 [3.2–3.8]
Ethnicity mother Caucasian	79 [86]*	78 [83]	53 [84]
Ethnicity father Caucasian	79 [86]*	80 [85]	53 [84]
Heredity: mother and/or father with			
Asthma	33 [35]*	38 [40]	10 [16]
Eczema	21 [23]*	22 [23]	8 [13]
Pollen allergy	52 [56]*	55 [58]	18 [29]
Duration of smoking in pregnancy months	9 [10]*	8 [8]	3 [5]
Current smoking	17 [18]*	19 [20]	12 [20]
Dog and/or cat at home	20 [22]*	20 [21]	13 [21]
Eczema	18 [20]*	22 [23]	3 [5]
Sensitised[#]	23 [26] [#]	27 [29] [#]	10 [20] [#]
>6 colds·year⁻¹	62 [67]*	61 [64]	14 [22]
Total WBC ×10⁹ cells·L⁻¹	11.4 [8.4–14.1] [§]	9.1 [7.2–11.2] ^f	8.8 [7.0–10.4]
Neutrophils ×10⁹ cells·L⁻¹	7.3 [4.1–10.4] [§]	3.3 [2.2–4.2] ^f	2.7 [1.9–3.7]
Eosinophils[¶] ×10⁹ cells·L⁻¹	0.0 [0.0–0.1]	0.3 [0.2–0.6]	0.2 [0.2–0.3]
Oral steroids at inclusion	86 [80]		0
25-OH-vitamin D		84 [67–99] ^{##}	83.5 [70.5–100.5] ^{¶¶}

Data are presented as n, n (%) or median (interquartile range), unless otherwise stated. [#]: specific IgE >0.35 kU·L⁻¹ in fx5 and/or phadiatop, n=87 analysed in the acute wheeze group, n=92 in the revisit group and n=49 controls; [¶]: eosinophil measurements missing for acute wheeze n=6, revisit n=3 and control n=4; *: 15 children have missing data, since they did not come to the follow-up visit after the acute visit; [§]: blood count missing n=6; ^f: blood count missing n=1; ^{##}: vitamin D level missing n=30; ^{¶¶}: vitamin D level missing n=12. WBC: white blood cells.

FIGURE 3 Characterisation of the acute wheezing children by comparison to healthy controls and the same children at revisit after 2–3 months. Characterisation by comparison of differentially expressed genes (DEGs), which were consistently a) upregulated in acute wheeze and b) downregulated in acute wheeze, when compared to healthy controls and their own revisit. Euler diagrams illustrate overlap of the DEGs. Enrichment of the consistently regulated genes in gene ontology biological process was tested using EnrichR, and the 10 most enriched terms based on the combined score were shown here with the significance (x-axis) and the overlap rate; the denominator is member genes of the category and the numerator is the consistent DEGs overlapping with the member genes of the category. CTRL: controls; ACW: acute wheeze; REV: revisit; ER: endoplasmic reticulum.

processes similar to the genes consistently upregulated in both comparisons, supporting the importance of interferons/cytokines in the wheezing preschool child.

WGCNA reveals an acute wheeze-specific co-regulated gene module

In the group-wise comparisons of differentially expressed genes we assumed homogenous characteristics within the groups. This is the most commonly used approach, but it tends to ignore genes that vary between individuals within a group. The dendrogram in figure 2c illustrates the individual variation within each group. The acute wheeze samples differed the most from the others, and the majority of the acute wheeze samples clustered together, but several of them were included in the cluster dominated by control samples. Based on that, we hypothesised that the transcriptomic profiles were heterogeneous even within each group, and that this variation might be relevant to their clinical characteristics. WGCNA was used to find modules of highly correlated genes within each sample group [22]. From 3079 significantly variable protein coding genes, three overlapping modules from co-regulated gene networks were defined in the acute wheeze, revisit and control sample groups (figure 2a, supplementary figures E2–E4 and supplementary table E3). We denote these gene networks by colour (blue, brown, turquoise, yellow and grey). In addition, one unique module consisting of 145 genes was defined for acute wheeze (ACW-yellow). We focused on that acute wheeze-specific module and used WGCNA [22] to identify clinical traits that correlated with this gene module.

Gene set enrichment analysis suggests variation in IFN responses in the acute-phase wheezing children

To reveal the roles of the 145 genes in the acute wheeze-specific yellow gene module, we applied gene set enrichment analysis. Genes for IFN-I/-II signalling pathways and responses, cytokine-mediated signalling pathways and antiviral response were the most enriched (figure 4a). Most prominent was the enrichment of genes in the IFN-signalling pathways and response. We found both genes crucial for signal transduction (e.g. *STAT1* and *STAT2*) as well as downstream IFN-sensitive response element (ISRE)-regulated antiviral genes (e.g. *OAS1*, *OAS2*, *OASL*, *MX1*) and γ-activated sequence (GAS)-regulated pro-inflammatory genes

FIGURE 4 Characterisation of a co-regulated gene module “ACW-yellow” specifically identified in acute wheezing children. a and b) Characterisation of ACW-yellow module by enrichment of the module genes in a) Gene Ontology Biological Process and b) Drug Perturbation from Gene Expression Omnibus (GEO) using human blood samples. Enrichments were tested by EnrichR, and the 10 most enriched terms on the combined score were shown here with the significance (x-axis) and the overlap rate; the denominator is member genes of the term, and the numerator is the ACW-yellow genes overlapping with the member genes. c) Significant associations between clinical traits and the ACW-yellow module. Each panel illustrates an association between the i) binary and ii) quantitative traits and the ACW-yellow modules. Each column is a

clinical trait. Each cell is coloured by $-\log_{10}(p) \times \text{sgn}(r)$, where p is p -value of the corresponding correlation, and r is the correlation coefficient; thus red is positive correlation and blue is negative. *: $p < 0.05$, #: $p < 0.005$, !: $p < 0.0005$. Legend of the trait IDs is in supplementary table E1, and the complete results are found in supplementary figures E5 and E6. d) Differential gene expression (y-axis) of *TRIM22*, a member of the acute wheeze-specific module, in the cases with (Yes) or without (No) a leukotriene receptor antagonist (LTRA) medication (montelukast) the year preceding the visit at 7 years of age (x-axis). Significance level (p -value at the top of each panel) was tested by Kruskal–Wallis rank sum test. e) Relationship between ACW-yellow eigengene and 7Y_LTRA. The probability at 7 years based on the expression profile at acute visit was modelled by logistic regression (table 3). Line is a trace of the probability (y-axis) according to ACW-yellow eigengene (x-axis). Point is a predicted probability (y-axis) of a child with acute wheeze based on the ACW-yellow eigengene (x-axis) with the prognosis (7Y_LTRA). f) Receiver–operating characteristic (ROC) curves and corresponding areas under the curve (AUC) statistics of the 7Y_LTRA risk prediction models. Dotted line is about the model using ACW-yellow eigengene only, and grey line is about ACW-yellow eigengene and ACW_WEIGHT. p -value was estimated by DeLong's test for two ROC curves. g) Relationship between ACW-yellow eigengene, ACW_WEIGHT and conditional probabilities of 7Y_LTRA. The probability at 7 years based on the expression profile and the body weight at the acute visit was modelled by multiple logistic regression (table 3). Line is a trace of the probability (y-axis) according to ACW-yellow eigengene (x-axis) in each body weight, either the first quartile (Q1), median or the third quartile (Q3). Point is a predicted probability (y-axis) of an ACW child based on the ACW-yellow eigengene (x-axis) and the body weight (coloured) with the prognosis. h) Negative correlation with *CREM* gene expression (y-axis) and 25-OH-vitamin D concentration of the cases at the first revisit (x-axis; RE_VITAMIND). Biweight midcorrelation coefficient (r) and the significance (p) are labelled.

(e.g. *IRF1*) to be enriched. Additionally, *USP18*, which mediates suppression of IFN-I signalling via STAT2 [23], is a member of this module. Genes within the IFN-III signalling pathway were not significantly enriched, probably due to the limited available information for this newest group of IFNs. The expression variation of IFNs (not of IFN-response genes) was not significant and therefore the IFNs were not included in WGCNA (out of the 3079 genes in figure 2a). Our results indicate that the acute wheeze-specific module represented a variation of IFN response for antiviral activity and inflammation in children with acute wheeze, but that IFN expression in peripheral leukocytes was independent of the IFN response in peripheral leukocytes.

To identify molecules that interfere with the genes in the acute wheeze-specific module, we again applied gene set enrichment analysis, but utilising a database focused on drug targets (figure 4b). We found that 52% (76 out of 145) of the acute wheeze-specific module genes were upregulated by *in-vitro* IFN- β stimulation of human peripheral blood mononuclear cells (PBMCs) (GEO:GSE26104, figure 4b). In addition, IFN-I/-II targets were significantly enriched, but the combined enrichment scores were relatively low. IFN-III targets could not be evaluated, as these were not available in the gene sets of the enrichment analysis. These results supported the contribution of IFN-I/-II in variation of acute wheeze, but did not exclude the contribution of IFN-III. Furthermore, an enrichment of genes downstream of lipopolysaccharide stimulation was found. In contrast, 31% (46 out of 145) of the acute wheeze-specific module genes were downregulated by etanercept, a known tumour necrosis factor inhibitor, or *in vitro* nitric oxide stimulation of cells (GEO:GSE13887, figure 4b).

Gene module–trait association analysis suggests that higher IFN response at the acute-phase wheezing is a predictor of poor respiratory prognosis

We used WGCNA [22] to identify clinical traits that correlated with the gene modules. 76 clinical traits (supplementary table E1) and blood cell counts were compared with representative expression patterns of each gene module (figure 4c, supplementary figures E5 and E6). The acute wheeze-specific module showed positive correlations with the FEV₁/FVC ratio at the visit at age 7 years (7Y_FEV₁%_FVC_RATIO in figure 4c; $p < 0.05$) and the leukotriene receptor antagonist (LTRA, montelukast) medication in the year preceding the revisit at 7 years (7Y_LTRA in figure 4c; $p < 0.005$). More than half of the acute wheeze-yellow module genes (55%; 80 out of 145) were significantly correlated with LTRA medication (adjusted $p < 0.05$; supplementary table E4), with *TRIM22* as the most significant gene (figure 4d; $p = 2.49 \times 10^{-4}$). *TRIM22* expression differed between children with or without LTRA treatment, with higher expression in those who received treatment. *TRIM22* is induced by IFN-I/-II/-III, and represses virus replication [24]. Among the 25 acute wheeze-specific module genes involved in the IFN-I-signalling pathway (figure 4a; GO:0060337), 19 (76%) genes including *TRIM22* were correlated with LTRA treatment. Similarly, 10 (59%) out of 17 genes of the IFN-II-mediated signalling pathway in this acute wheeze-specific module were also correlated with LTRA treatment at 7 years. The differential expression of *TRIM22* seems to be independent of allergies in our material (supplementary figures E7a).

Based on the WGCNA analyses, we used logistic regression to develop a prediction model, and the acute wheeze-specific gene module was shown to be a predictor of LTRA-medication at 7 years with high accuracy (table 3; area under the curve (AUC)_{ACW-yellow} 0.785, 95% CI 0.652–0.917; figure 4e and f). Among the correlated traits in WGCNA, the body weight at acute visit was suggested as the primary confounder (table 3; AUC_{ACW-yellow+weight} 0.815, 95% CI 0.668–0.962; figure 4g), and was therefore added to the prediction model, although no significant improvement of the prediction model was seen ($p = 0.77$; figure 4f). This effect by body weight could not be explained by the age or sex of the child (table 3; BG_GENDER and ACW_AGE in supplementary figures E5 and E6). The significant association between

TABLE 3 Logistic regression models for prediction of prognostic traits

	Coefficient _t ±se	Z-value	Pr(> z)	OR (95% CI)
Model for 7Y_LTRA prediction by ACW-yellow eigengene				
Intercept	-11.577±3.906			
ACW-yellow	9.675±3.668	2.638	0.008	1.592×10 ⁴ [2.004×10 ¹ –5.005×10 ⁷]
Model for 7Y_LTRA prediction by ACW-yellow eigengene and ACW_WEIGHT[#]				
Intercept	-9.725±4.251			
ACW-yellow	13.565±4.456	3.044	0.002	7.783×10 ⁵ [2.888×10 ² –1.639×10 ¹⁰]
ACW_WEIGHT	-0.532±0.227	-2.339	0.019	5.877×10 ⁻¹ [3.550×10 ⁻¹ –8.797×10 ⁻¹]
Model for 7Y_LTRA prediction by ACW-yellow eigengene, BG_GENDER and ACW_AGE[#]				
Intercept	-1.412×10 ¹ ±4.278×10 ¹			
ACW-yellow	9.523×10 ⁰ ±3.718×10 ⁰	2.561	0.010	
BG_GENDER	1.172×10 ⁻¹ ±6.974×10 ⁻¹	0.246	0.806	
ACW_AGE	6.517×10 ⁻⁵ ±1.058×10 ⁻³	0.062	0.951	
Model for 7Y_LTRA prediction by ACW-yellow eigengene, ACW_WEIGHT and REV_VITAMIND[#]				
Intercept	-10.528±6.002			
ACW-yellow	13.988±5.592	2.501	0.012	
ACW_WEIGHT	-0.465±0.236	-1.975	0.048	
REV_VITAMIND	-0.004±0.021	-0.182	0.856	
Model for 7Y_ASTHMA_GA2LEN prediction by REV-turquoise eigengene				
Intercept	9.688±3.518	2.754		
REV-turquoise	-8.323±3.355	-2.481	0.013	2.429×10 ⁻⁴ [1.852×10 ⁻⁷ –1.162×10 ⁻¹]

In all models, subjects with missing values, were excluded before the model fitting. LTRA: leukotriene receptor antagonist; ACW: acute wheeze. #: there was no multiple colinearity between the explanatory variables.

the acute wheeze-specific yellow module and clinical traits suggested that children with higher IFN response at acute wheeze often had more LTRA-responding respiratory symptoms at the age of 7 years.

The acute wheeze-specific module showed a negative correlation with the number of visits to the emergency room between acute-visit and revisit ($p<0.05$; figure 4c), and 25-OH vitamin D levels in the cases (REV_VITAMIND in figure 4c; $p<0.005$). Vitamin D levels were not a confounder of the prediction model for LTRA treatment at 7 yrs (table 3). 51 (35%) out of the 145 acute wheeze-specific module genes were significantly correlated with vitamin D levels (adjusted $p<0.05$; supplementary table E5), with *CREM* the most significant gene (figure 4h; $p=4.12\times10^{-4}$). Of these 51 genes, 38 genes (75%, including *STAT1*, *TRIM22* and *CREM*) were also correlated with LTRA at the age of 7 years, suggesting association between vitamin D level and the prognosis. In the acute wheeze-specific gene module, 10 (40%) of the 25 genes in the IFN-I-signalling pathway, and eight (47%) of the 17 genes in the IFN- γ -mediated signalling pathway were correlated with the vitamin D levels at revisit.

Gene module-trait association analysis suggests that downregulation of the turquoise module at the revisit indicates an increased risk for allergic asthma and less asthma control at the age of 7 years

The REV-turquoise gene module showed association to asthma-related traits (figure 5a). The module was associated to asthma (7Y_ASTHMA_GA2LEN), allergic asthma (7Y_ASTHMA_ALLERGIC) and asthma control (7Y_ASTHMA_CONTROL_TEST), all at 7 years of age. By logistic regression analysis, this module was a predictor of prognostic asthma risk (7Y_ASTHMA_GA2LEN; table 3, figure 5b and c, AUC 0.730, 95% CI 0.573–0.888). Among the REV-turquoise genes, downregulation of *DYNC1I2* at the revisit was the most significant in asthma at 7 years (figure 5d), and *PRMT9* for allergic asthma at 7 years (figure 5e and f). In this module there were genes for targeting of proteins to the endoplasmic reticulum, nonsense-mediated mRNA degradation and viral transcription and processes enriched (figure 5g). The REV-turquoise module consists of 1257 genes (supplementary figure E4). <10% of the module genes (118 out of 1257) were found in the enrichment analysis of the module, and therefore the annotation was limited. Because of a correlation to lymphocyte counts at the revisit (figure 5a and h, supplementary figure E7B), the reason for the co-expression of the module genes probably represent changes of the lymphocyte population in the

peripheral blood leukocytes rather than changes of a specific molecular pathway. Therefore, our data indicate that downregulation of the module genes by lower lymphocyte count at the revisit suggest higher risk for allergic asthma, and progress to chronic asthma.

Discussion

The most prominent findings of the significant differences in gene expression between wheezing children and healthy controls showed upregulation of genes involved in IFN response, as well as neutrophil activity. Furthermore, we identified an acute wheeze-specific gene module consisting of 145 genes, which was associated to vitamin D levels in infancy, as well as with FEV₁%/FVC and asthma medication several years later. This module was a predictor of asthma medication at 7 years with high accuracy, and therefore genes in this module are strong candidates as prognostic markers. The major role of the genes in this module was related to the innate immune response combined with reduction of viral replication through IFN signalling. Another co-regulated gene module correlated to development of allergic asthma.

Interferons are secreted as part of the innate immune response by lymphocytes and infected non-immune cells, *e.g.* epithelial cells, as defence against pathogens. Viral infections trigger as many as 85% of acute asthma attacks in children, with rhinovirus as the most common agent [25]. During a respiratory viral infection, an increase of IFN-I and -III will occur, followed by induction of, *e.g.* cytokines and accumulation of immune cells, but the specific response differs depending on age and the viral trigger [9, 26, 27]. Rhinovirus-associated acute wheeze correlates with higher serum IFN-II levels than respiratory syncytial virus-associated acute wheeze [27]. Similarly, an *in vitro* approach revealed that IFN- α /- β /- λ are produced by rhinovirus infection in bronchial epithelial cells and PBMCs [28]. These studies suggest an increased serum IFN level in children with an acute viral infection with wheeze. Furthermore, a recent observation showed that the general impairment of IFN-I/-III production in asthmatics previously seen both *in vivo* [10] and *in vitro* [29–31] can be overcome at an acute rhinovirus infection [10]. Our study consistently revealed an enrichment of genes known to be upregulated by IFN-stimulation in the acute wheeze-specific module. Genes crucial for signal transduction, as well as downstream ISRE-regulated antiviral genes, and GAS-regulated pro-inflammatory genes were enriched, clearly showing an increased IFN response in acute wheeze. Importantly, this gene module identified in infancy was significantly correlated with LTRA medication at 7 years. In common Swedish practice, LTRA is used as add-on medication to inhaled corticosteroids. Based on this, our data suggested that children with higher serum IFN level at acute wheeze are those with the worst prognosis, as they tend to need continuous LTRA medication several years later.

Decreased IFN levels in asthmatic samples [10, 29–31] and low IFN- γ production in the first year of life have been suggested as predictors of childhood wheeze [32]. LTRA blocks leukotrienes and increases IFN- γ production in T-lymphocytes [33]. LTRA medication will support the native IFN production to prevent infection as an asthma trigger, or asthma exacerbations. We hypothesise that the children in our cohort in general, but especially those with LTRA treatment at 7 years, were those with a generally impaired IFN production at an early age. Therefore, those children were more severely affected by common respiratory viruses, such as rhinovirus, resulting in acute wheeze severe enough to result in a visit to the emergency room at the hospital at an early age. However, the reason for this suggested impairment is unknown. For the majority of infants with wheeze it is a transient condition, and viral-induced wheezing will remit by school age [34]. At least part of the explanation might be that IFN- γ production increases with age [35]. Wheeze caused by early rhinovirus infections has been associated with a significantly increased risk of asthma later in childhood [1], and it was suggested recently [26] that identification of the viral trigger should be included in the diagnosis of bronchiolitis, to improve treatment. In our cohort, rhinovirus was detected in 74% at acute wheeze, dominated by species rhinovirus-C, and children with species-specific IgG₁ antibody increase against rhinovirus-A or against rhinovirus-A and -C until the revisit had longer time with respiratory symptoms [13]. As IFN- γ reduces IgG₁ production [36, 37], rhinovirus-A could result in lower IFN- γ induction than the other rhinovirus subtypes. Importantly, the time with reported respiratory symptoms seemed to relate more to the antibody response than the rhinovirus species at the acute visit [13], indicating a more general deficiency of the early innate immune response.

Vitamin D is required for IFN-mediated defence against pathogens; its deficiency is a risk factor for childhood asthma, and vitamin D supplementation during pregnancy seems to reduce risk of asthma/recurrent wheeze in the offspring [38–41]. In this cohort, we have shown previously that low vitamin D (25-OH)D (<30 ng·mL⁻¹) is associated with an increased risk of acute wheeze [12]. Thus, either reduced IFN production capacity or vitamin D deficiency, or both, could lead to viral-induced exacerbation through deficient IFN response. Our data imply that vitamin D-deficient children tend to be on LTRA medication at age 7 years for prevention/alleviation of respiratory symptoms. Our study not only supports the impact of vitamin D levels for wheeze, but also shows a connection to gene expression, with *CREM* as the most negatively correlated gene. *CREM* is a repressor of the vitamin D receptor expression [42], and a negative regulator of the T-helper type 2 response [43]. Interestingly, the expression of the vitamin D receptor may be a limiting factor in the vitamin D response. A general deficiency of the innate immune response in children with rhinovirus-induced respiratory symptoms would also provide room for other viruses and even bacteria to colonise and affect the host. In our cohort, children with vitamin D

insufficiency were not colonised with virus or bacteria to any higher degree than vitamin D-sufficient children [12].

Another interesting finding was that downregulation of the REV-turquoise module genes at the revisit indicates an increased risk for allergic asthma, and less asthma control several years later, at the age of 7 years. The risk of adverse health effects in the offspring by maternal tobacco smoking during pregnancy is well documented [44], with for example increased risk of childhood wheeze and asthma [45], but its effect on development of allergic sensitisation in the child is unclear. In our material, a correlation was seen between the module genes and smoking of the mother during pregnancy and allergic sensitisation during infancy. In a previous study, >80% of Swedish school children with problematic severe asthma or persistent asthma were atopic [46]. Allergic asthma is often chronic and requires anti-inflammatory treatment, which would be represented by the correlation of the gene module with asthma control at 7 years in our material. Based on our data, the REV-turquoise module genes could be evaluated as biomarkers for development of allergic asthma. This gene module may serve as a complement to clinical follow-ups to identify the preschool wheezing children that will gain the most from more frequent clinical examinations as well as more active anti-inflammatory treatment. Unlike the prediction value of the acute wheeze-specific gene module which is limited to sampling during acute wheeze, the turquoise module could be evaluated at any timepoint without requirements of ongoing respiratory symptoms.

Our study had some limitations and the findings have to be further validated in independent longitudinal cohorts. Many genes show cell-type specific expression patterns, and therefore it is preferable to study gene expression in disease-relevant tissue whenever possible. However, disease-relevant tissue can be very hard or impossible to access, especially when investigating diseases in preschool children. As acute wheezing primarily affects the airways, the most relevant samples would be bronchial epithelial cells or bronchial biopsies, which is not realistic in studies of preschool children. Blood is more accessible, as it demands less invasive sampling and reflects relevant changes in the regulation of the inflammatory mechanisms. Despite the heterogeneity in the samples, our analyses approach of the transcriptome in peripheral blood identified an acute wheeze-specific gene module, that associated with later clinical characteristics of the wheezing children. Gene enrichment analyses showing plausible pathways, supported our approach of studying expression profiling for respiratory diseases in peripheral blood. To increase the molecular understanding of the pathophysiology and identification of therapeutic targets, further mechanistic studies will be necessary.

In conclusion, our data strongly support the role of skewed IFN-response in children with wheeze. Despite the heterogeneity in the acute wheeze samples, the gene expression changes in the acute-wheeze specific module correlated with lung-function measurements at 7 years of age and LTRA treatment in the year preceding the revisit at age 7 years. This acute wheeze-specific gene module was a predictor of asthma medication with high accuracy, and therefore we hypothesise that these 145 genes are candidate diagnostic, or even prognostic, markers for a more long-term prognosis of preschool children with wheeze. The gene expression profiles in this acute wheeze group may reflect the cause of the wheezing, and could in that case, serve as candidate markers for more short-term outcomes. Moreover, the same analysis approach might well identify relations between other variable gene modules and clinical characteristics. This gives hope to the long-term aim of development of trustworthy prognostic models based on blood biomarkers.

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Author contributions: S. Katayama designed, developed and performed statistical and bioinformatic analyses. K. Stenberg Hammar and G. Hedlin, clinical characterisation. K. Krjutškov developed lab procedures and generated the data by RNA-seq. E. Einarsson contributed to the bioinformatic analysis. J. Kere conceived the study together with C. Söderhäll and supervised transcriptome analyses. C. Söderhäll conceived the study and coordinated the analyses and the project. S. Katayama, K. Stenberg Hammar, K. Krjutškov, E. Einarsson, G. Hedlin, J. Kere and C. Söderhäll contributed to the interpretation of the data. S. Katayama and C. Söderhäll wrote the manuscript, and all authors read, edited and approved the manuscript.

Conflict of interest: S. Katayama has nothing to disclose. K. Stenberg Hammar has nothing to disclose. K. Krjutškov has a patent "GlobinLock – blocking oligonucleotides" pending. E. Einarsson has nothing to disclose. G. Hedlin has nothing to disclose. J. Kere has acted as scientific advisor for Blueprint Genetics, Inc., Helsinki, Finland, outside the submitted work; and has a patent GlobinLock method pending. C. Söderhäll has nothing to disclose.

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References

- 1 Jackson DJ, Gangnon RE, Evans MD, *et al.* Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children. *Am J Respir Crit Care Med* 2008; 178: 667–672.
- 2 Bønnelykke K, Vissing NH, Sevelsted A, *et al.* Association between respiratory infections in early life and later asthma is independent of virus type. *J Allergy Clin Immunol* 2015; 136: 81–86.
- 3 Silvestri M, Franchi S, Pistorio A, *et al.* Smoke exposure, wheezing, and asthma development: a systematic review and meta-analysis in unselected birth cohorts. *Pediatr Pulmonol* 2015; 50: 353–362.
- 4 Rubner FJ, Jackson DJ, Evans MD, *et al.* Early life rhinovirus wheezing, allergic sensitization, and asthma risk at adolescence. *J Allergy Clin Immunol* 2017; 139: 501–507.
- 5 Ivashkiv LB, Donlin LT. Regulation of type I interferon responses. *Nat Rev Immunol* 2014; 14: 36–49.
- 6 Moro K, Kabata H, Tanabe M, *et al.* Interferon and IL-27 antagonize the function of group 2 innate lymphoid cells and type 2 innate immune responses. *Nat Immunol* 2016; 17: 76–86.
- 7 de Weerd NA, Nguyen T. The interferons and their receptors – distribution and regulation. *Immunol Cell Biol* 2012; 90: 483–491.
- 8 Jartti T, Gern JE. Role of viral infections in the development and exacerbation of asthma in children. *J Allergy Clin Immunol* 2017; 140: 895–906.
- 9 Restori KH, Srinivasa BT, Ward BJ, *et al.* Neonatal immunity, respiratory virus infections, and the development of asthma. *Front Immunol* 2018; 9: 1249.
- 10 Bergauer A, Sopel N, Kroß B, *et al.* IFN- α /IFN- λ responses to respiratory viruses in paediatric asthma. *Eur Respir J* 2017; 49: 1700006.
- 11 Gelfand EW, Schedel M. Molecular endotypes contribute to the heterogeneity of asthma. *Immunol Allergy Clin North Am* 2018; 38: 655–665.
- 12 Stenberg Hammar K, Hedlin G, Konradsen JR, *et al.* Subnormal levels of vitamin D are associated with acute wheeze in young children. *Acta Paediatr* 2014; 103: 856–861.
- 13 Stenberg-Hammar K, Niespodziana K, Söderhäll C, *et al.* Rhinovirus-specific antibody responses in preschool children with acute wheeze reflect severity of respiratory symptoms. *Allergy* 2016; 71: 1728–1735.
- 14 Sundbom F, Lindberg E, Bjerg A, *et al.* Asthma symptoms and nasal congestion as independent risk factors for insomnia in a general population: results from the GA²LEN survey. *Allergy* 2013; 68: 213–219.
- 15 Liu AH, Zeiger R, Sorkness C, *et al.* Development and cross-sectional validation of the Childhood Asthma Control Test. *J Allergy Clin Immunol* 2007; 119: 817–825.
- 16 Krjutškov K, Katayama S, Saare M, *et al.* Single-cell transcriptome analysis of endometrial tissue. *Hum Reprod* 2016; 31: 844–853.
- 17 Krjutškov K, Koel M, Roost AM, *et al.* Globin mRNA reduction for whole-blood transcriptome sequencing. *Sci Rep* 2016; 6: 31584.
- 18 Katayama S, Skoog T, Söderhäll C, *et al.* Guide for library design and bias correction for large-scale transcriptome studies using highly multiplexed RNAseq methods. *BMC Bioinformatics* 2019; 20: 418.
- 19 Katayama S, Töhönen V, Linnarsson S, *et al.* SAMstr: statistical test for differential expression in single-cell transcriptome with spike-in normalization. *Bioinformatics* 2013; 29: 2943–2945.
- 20 Li J, Tibshirani R. Finding consistent patterns: a nonparametric approach for identifying differential expression in RNA-Seq data. *Stat Methods Med Res* 2013; 22: 519–536.
- 21 Kuleshov MV, Jones MR, Rouillard AD, *et al.* Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res* 2016; 44: W90–W97.
- 22 Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics* 2008; 9: 559.
- 23 Arimoto KI, Löchte S, Stoner SA, *et al.* STAT2 is an essential adaptor in USP18-mediated suppression of type I interferon signaling. *Nat Struct Mol Biol* 2017; 24: 279–289.
- 24 Lian Q, Sun B. Interferons command Trim22 to fight against viruses. *Cell Mol Immunol* 2017; 14: 794–796.
- 25 Gern JE. The ABCs of rhinoviruses, wheezing, and asthma. *J Virol* 2010; 84: 7418–7426.
- 26 Jartti T, Smits HH, Bønnelykke K, *et al.* Bronchiolitis needs a revisit: distinguishing between virus entities and their treatments. *Allergy* 2019; 74: 40–52.
- 27 Jartti T, Paul-Anttila M, Lehtinen P, *et al.* Systemic T-helper and T-regulatory cell type cytokine responses in rhinovirus vs. respiratory syncytial virus induced early wheezing: an observational study. *Respir Res* 2009; 10: 85.
- 28 Khaitov MR, Laza-Stanca V, Edwards MR, *et al.* Respiratory virus induction of alpha-, beta- and lambda-interferons in bronchial epithelial cells and peripheral blood mononuclear cells. *Allergy* 2009; 64: 375–386.
- 29 Wark PA, Johnston SL, Bucchieri F, *et al.* Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. *J Exp Med* 2005; 201: 937–947.
- 30 Contoli M, Message SD, Laza-Stanca V, *et al.* Role of deficient type III interferon- λ production in asthma exacerbations. *Nat Med* 2006; 12: 1023–1026.
- 31 Zhu J, Message SD, Mallia P, *et al.* Bronchial mucosal IFN- α / β and pattern recognition receptor expression in patients with experimental rhinovirus-induced asthma exacerbations. *J Allergy Clin Immunol* 2019; 143: 114–125.
- 32 Stern DA, Guerra S, Halonen M, *et al.* Low IFN- γ production in the first year of life as a predictor of wheeze during childhood. *J Allergy Clin Immunol* 2007; 120: 835–841.
- 33 Spinozzi F, Russano AM, Piattoni S, *et al.* Biological effects of montelukast, a cysteinyl-leukotriene receptor-antagonist, on T lymphocytes. *Clin Exp Allergy* 2004; 34: 1876–1882.

- 34 Martinez FD, Wright AL, Taussig LM, *et al.* Asthma and wheezing in the first six years of life. *N Engl J Med* 1995; 332: 133–138.
- 35 Gasparoni A, Ciardelli L, Avanzini A, *et al.* Age-related changes in intracellular TH1/TH2 cytokine production, immunoproliferative T lymphocyte response and natural killer cell activity in newborns, children and adults. *Biol Neonate* 2003; 84: 297–303.
- 36 Snapper CM, Paul WE. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* 1987; 236: 944–947.
- 37 Berton MT, Uhr JW, Vitetta ES. Synthesis of germ-line $\gamma 1$ immunoglobulin heavy-chain transcripts in resting B cells: induction by interleukin 4 and inhibition by interferon γ . *Proc Natl Acad Sci USA* 1989; 86: 2829–2833.
- 38 Fabri M, Stenger S, Shin D-M, *et al.* Vitamin D is required for IFN- γ -mediated antimicrobial activity of human macrophages. *Sci Transl Med* 2011; 3: 101ra02.
- 39 Litonjua AA. Vitamin D and childhood asthma: causation and contribution to disease activity. *Curr Opin Allergy Clin Immunol* 2019; 19: 126–131.
- 40 Arikoglu T, Kuyucu S, Karaismailoglu E, *et al.* The association of vitamin D, cathelicidin, and vitamin D binding protein with acute asthma attacks in children. *Allergy Asthma Proc* 2015; 36: 51–58.
- 41 Feng H, Xun P, Pike K, *et al.* In utero exposure to 25-hydroxyvitamin D and risk of childhood asthma, wheeze, and respiratory tract infections: a meta-analysis of birth cohort studies. *J Allergy Clin Immunol* 2017; 139: 1508–1517.
- 42 Huening M, Yehia G, Molina CA, *et al.* Evidence for a regulatory role of inducible cAMP early repressor in protein kinase A-mediated enhancement of vitamin D receptor expression and modulation of hormone action. *Mol Endocrinol* 2002; 16: 2052–2064.
- 43 Verjans E, Ohl K, Reiss LK, *et al.* The cAMP response element modulator (CREM) regulates TH2 mediated inflammation. *Oncotarget* 2015; 6: 38538–38551.
- 44 US Department of Health and Human Services. The Health Consequences of Smoking: 50 Years of Progress. A Report of the Surgeon General. Atlanta, U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health, 2014.
- 45 McEvoy CT, Spindel ER. Pulmonary effects of maternal smoking on the fetus and child: effects on lung development, respiratory morbidities, and life long lung health. *Paediatr Respir Rev* 2017; 21: 27–33.
- 46 Konradsen JR, Nordlund B, Lidegran M, *et al.* Problematic severe asthma: a proposed approach to identifying children who are severely resistant to therapy. *Pediatr Allergy Immunol* 2011; 22: 9–18.