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Gene expression in CD4+ T-cells reflects heterogeneity in infant wheezing phenotypes

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ABSTRACT: Although a marked increase in the reporting of wheezing symptoms since the mid-1970s has been described, the underlying immunopathology of the different wheezing phenotypes has not been clarified. Since differences in gene expression might be involved, the objective of the present study was to identify gene expression profiles in CD4+ T-cells from two distinct infant wheezing phenotypes.

The gene expression profiles of peripheral CD4+ T-cells were compared by means of microarray analysis of six transient wheezers, six persistent wheezers and seven healthy controls. The differentially expressed genes were subsequently validated by RT-PCR.

The differential gene expression profiles reflected common immunological pathways involved in apoptosis or proliferation of T-cells. Furthermore, both wheezing phenotypes showed decreased expression of the complement component 5 receptor 1 gene, a gene involved in the regulation of bronchial responsiveness. Moreover, differences in gene expression profiles were found in genes involved in the immune response against respiratory syncytial virus, such as those encoding signal transducer and activator of transcription 1 and an inflammatory mediator showing enhanced production in asthma (prostaglandin E_2 receptor 2).

The present findings suggest that clinical symptoms of wheeze are reflected in common immunological pathways, whereas differences between wheezing phenotypes are, in part, reflected in distinct gene expression profiles.

KEYWORDS: Childhood, gene expression profiles, immunopathology, microarray, wheezing phenotypes

It has been reported that the prevalence of wheezing symptoms in preschool children has increased since the mid-1970s [1]. This increase has been described in not only atopic children but also nonatopic children who only wheeze during a lower respiratory tract infection [2]. In addition, various studies have shown an increase in bronchial hyperresponsiveness and airway abnormalities without an associated change in prevalence of atopy [2–4]. In preschool children with wheezing symptoms, several distinct phenotypes have been described. The Tucson birth cohort study showed that a third of all newborns in the industrialised world experience one or more episodes of wheezing during the first 3 yrs of life, mostly associated with viral respiratory infections (early wheeze). Of these infants, 60% discontinue wheezing between the ages of 3 and 6 yrs (transient early wheeze; TW). The children who continue to wheeze (persistent wheeze; PW) are regarded as asthmatics [5]. To date, the change in prevalence of wheezing symptoms has mostly been attributed to environmental factors. However, knowledge of the underlying immunopathological mechanisms is lacking [3, 4, 6].

An immature immune system might explain the susceptibility of infants to the development of lower respiratory tract infection and subsequent wheezing [4]. In the immunopathology of asthma, the disease linked to PW, an important role has been described for CD4+ T-cells. These CD4+ T-cells are active at the local inflammatory sites, *i.e. via* the release of type-2 T-helper cell (Th2) cytokines [7]. Interestingly, infants show diminished Th1 and Th2 lineage cytokine responses to nonspecific stimuli (*e.g.* viruses) compared to adults [8]. The most extensively investigated virus associated with infant wheezing in the first year of life is respiratory syncytial virus (RSV). This virus

For editorial comments see page 1138.

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is the most common cause of acute airway obstruction and subsequent wheezing in infants [9, 10]. During RSV infection, children produce low levels of protective antibodies against this virus [11]. Interestingly, the magnitude of interferon (IFN)-γ production during RSV infection seems to be indicative of disease severity and predictive of the subsequent development of PW [12, 13]. Another IFN, IFN-α, also seems to play a role in determining the severity of a RSV infection, and subsequent wheezing, in infants [14].

Combining the immunological profile of an infant with genetic and environmental risk factors might give a good indication as regards which infants are at risk of developing PW, and might lead to new intervention strategies. Genetic studies have revealed several asthma susceptibility genes. For instance, the ADAM metallopeptidase domain 33 gene has been demonstrated to show linkage to atopic asthma, although its precise function has not yet been revealed [15]. Further immunology-related genes have also been linked to asthma and atopy, such as those encoding RANTES (regulated on activation, normal T-cell expressed and secreted; one of whose receptors is CC chemokine receptor 5) and complement component 5 (C5) [16, 17]. Although these studies are promising, the causative role of these genes in the pathophysiology of asthma, and especially childhood wheeze, remains to be identified. A promising technique for the unravelling of pathophysiological pathways in complex disease is the identification of gene expression profiles by microarray analysis, or genomics. For this technique, RNA from disease-specific tissue is required. Previous studies have shown that peripheral blood mononuclear cells (PBMCs) reflect disease-specific changes in an organ, and can be used as a model for the characterisation and monitoring of asthma using microarrays [18–20]. Unstimulated PBMCs most closely resembles the *in vivo* activation state of these cells [21]. Furthermore, the use of microarray analysis permits the investigation of a disease or process objectively without a stringent hypothesis [18, 22, 23].

The aim of the present study was to determine the genetic profiles of children with either a transient or a persistent form of wheezing by means of microarray analysis in order to determine the differential expression of genes in various immunological pathways and distinguish biomarkers in heterogeneous wheezing phenotypes.

METHODS

Patients and control subjects

The patients were 6-yr-old children with TW and PW who had previously participated in another study because of recurrent or chronic wheeze episodes at the age of 1–4 yrs [24]. Since they were included as having shown early wheeze, their history of wheezing was well documented and, therefore, these children could be identified as showing TW or PW at the age of 6 yrs. Since the earlier study represented more PW than TW, another TW group (n=2) was recruited from Isala Clinics (Zwolle, the Netherlands) using the same characterisation and inclusion criteria. At the time of blood collection, the mean$\pm$SD age of the healthy control (HC; n=7), TW (n=6) and PW group (n=8) was 6.0$\pm$0.5, 5.7$\pm$0.5 and 6.0$\pm$0.5 yrs, respectively. The sex ratios (male:female) were 4:3, 5:3 and 4:2 for the HC, PW and TW group, respectively (table 1). Parents were asked to fill in a standardised questionnaire, based on the International Study of Asthma and Allergies in Childhood questionnaire, in order to define the phenotype precisely [25]. The TW group were defined as not exhibiting any wheezing complaints after the age of 3 yrs and not in need of any medication in order to obtain this clinical state. The PW group were defined as still showing wheezing complaints after the age of 3 yrs, for which the use of medication and follow-up by a paediatrician or paediatric pulmonologist was required.

The aim of the present study was to find true differences between TW and PW that were not attributable to active inflammation. Since both the TW and HC group were not permitted to have any wheezing complaints, a physical examination was performed at the moment of venous blood sampling in order to rule out current wheeze. Blood samples were obtained only when the child was not wheezing and had not done so for the past 6 weeks for the PW group (indicating proper disease control and no active inflammation), and the past 3 yrs for the TW group. The HC group were 6-yr-old children undergoing surgery. One HC underwent an orthopaedic surgical procedure, whereas the other HC subjects underwent urological interventions. None of them had a history of wheezing, allergy or a recent infection, nor a first-degree family member with an allergy or asthma. Blood sampling took place in the operating room within 10 min after the induction of general anaesthesia in order to avoid any effect of anaesthetics on parameters of inflammation [26, 27]. The present study was approved by the Medical Ethics Committee of the University Medical Centre (Utrecht, the Netherlands) and Isala Clinics. Written informed consent was obtained from the parents.

Study design

The gene expression profiles of unstimulated peripheral CD4+ T-cells from children with either PW or TW were compared with those of HC subjects by means of microarray analysis.

CD4+ T-cell isolation and RNA extraction

Venous blood ($\geqslant$5 mL) was collected. In order to minimise differences in gene expression attributable to sample handling,

TABLE 1 Patient characteristics

	Healthy control	Persistent wheeze	Transient wheeze
Subjects	7	8	6
Age at inclusion yrs	6.0$\pm$0.5	6.0$\pm$0.5	5.7$\pm$0.5
Wheezing at age >3 yrs	0/7	8/8	0/6
Medication[#]			
Bronchodilators	0/7	7/8	1/6
Steroids	0/7	6/8	0/6
Atopy	0/7	3/8	1/6
Eczema	0/7	3/8	3/6
Neither atopy nor eczema	7/7	2/8	3/6
Family history of atopy/asthma[¶]	0/7	5/7[+]	4/6

Data are presented as mean$\pm$SD or absolute numbers. [#]: use at time of blood sampling; [¶]: in first-degree relatives; [+]: data missing for one patient.

all samples were processed within 2 h of collection and handled in exactly the same manner. In the HC group, samples were collected within 15 min of application of general anaesthetics. CD4+ T-cells were isolated by means of Ficoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation and immunomagnetic separation as described previously [21]. The CD4+ T-cells were $\geqslant$95% pure as assessed by fluorescence-activated cell sorter analysis.

Total RNA was isolated by using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The quality of the RNA was assessed on the basis of demonstration of distinct 23s and 18s ribosomal RNA bands following electrophoresis on an agarose gel using a bioanalyser (Agilent 2100 Bioanalyzer; Agilent Technologies, Palo Alto, CA, USA) according to the manufacturer's protocol and a spectrophotometric ratio of absorbance at 260 nm to that at 280 nm of >1.8.

Sample handling and microarrays

RNA was submitted to the GeneChip® Core Facility (Affymetrix, High Wycombe, UK). Before hybridisation, the RNA was purified using RNeasy columns (Qiagen, Hilden, Germany). Subsequently, a total of 100 ng purified RNA per sample was used in the GeneChip® two-cycle complementary DNA (cDNA) synthesis kit provided by Affymetrix (High Wycombe). Then the cDNA was applied to the HG-U133A GeneChip® (Affymetrix, Santa Clara, CA, USA) according to Affymetrix guidelines [28]. In order to determine individual expression levels, one chip was used per sample and no samples were pooled.

Microarray data analysis

The scanned output files of the Affymetrix data set were analysed using Microarray Suite 5.0 software (Affymetrix, High Wycombe). Second-stage data analysis was performed using ArrayAssist software (Lobion Laboratories, Stratagene, La Jolla, CA, USA), using probe logarithmic intensity error (PLIER) estimate significance analysis, which produces an improved signal by accounting for observed patterns of probe behaviour on a chip [29]. The minimal fold-change rate was set at 1.5, with a p-value of $\leqslant$0.05 [30, 31]. All of the genes within these limits were considered to be of interest. Expressed sequence tags, hypothetical genes and genes of unknown function were omitted from the list.

RT-PCR analyses

For semi-quantitative RT-PCR analysis of the genes detected by microarray analysis, the same samples were used as in the microarray analysis. However, the RNA samples of three HC, one TW and one PW subjects were no longer available for RT-PCR analysis. Two additional new PW samples were added, derived from the same local study. Unfortunately, no additional samples were available for the TW or HC groups. A total of 100 ng total RNA from seven PW, five TW and four HC subjects were used for cDNA synthesis, using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Primers (Sigma-Genosys, The Woodlands, TX, USA) were designed using Primer3 software from the Whitehead Institute/MIT Center for Genome Research (Cambridge, MA, USA) [32]. RT-PCR analysis was performed using the MyiQ™ single-colour real-time PCR (Bio-Rad) as described previously. Gene expression data are provided relative to the lowest expression in one of the three groups.

Statistical analysis

Basic descriptive statistics were used to describe patient characteristics. For microarray data analysis, a PLIER algorithm, from ArrayAssist (Stratagene), was used with a p-value of $\leqslant$0.05 [29].

RESULTS

Identification of genes differentially regulated in both wheezing phenotypes

The gene expression profiles of six PW and six TW patients were compared with those of seven HC subjects. A total of 67 genes were found to be significantly differentially expressed in both wheezing groups using the PLIER method after deletion of all genes of unknown function, hypothetical genes and expressed sequence tags. Increased expression of 45 genes and decreased expression of 22 genes was found in the wheezing phenotypes (tables 2 and 3).

Validation of genes by semi-quantitative RT-PCR reveals two immunological pathways

A total of seven immune-related genes were randomly selected for validation by semi-quantitative RT-PCR. Using semi-quantitative RT-PCR, the increased expression of the heat shock 70 kDa protein 1A gene (*HSPA1A*) and decreased expression of the genes encoding complement component 5 receptor 1 (*C5R1*), Jun B proto-oncogene (*JUNB*), tumour necrosis factor-α-induced protein 3 (*TNFAIP3*), dual specificity phosphatase 2 (*DUSP2*), leukocyte immunoglobulin (Ig)-like receptor, subfamily B, member 2 (*LILRB2*) and tumour necrosis factor (ligand) superfamily, member 13b (*TNFSF13B*) were confirmed. The group of genes that displayed decreased expression contained a subgroup of genes involved in apoptosis or proliferation of T-cells.

Identification of a distinct gene expression profile in transient wheeze

In the TW group, six individual samples were compared with those from seven HC subjects and subsequently six PW patients. In these individual TW samples, the expression of 34 genes was $\geqslant$1.5-fold different from that in the HC group. Of these 38 genes, six were found to be upregulated, whereas 28 were downregulated (table 4). Among the 10 upregulated genes, three were immune-related. Furthermore, three different transcripts of one gene, encoding glutathione-*S*-transferase M1 (*GSTM1*), were consistently upregulated. Among the downregulated genes, 17 were immune-related, whereas the others were involved in either protein folding and transportation or regulation of RNA expression. Amongst the downregulated immune-related genes, two different transcripts of the gene encoding signal transducer and activator of transcription 1 (*STAT1*) were consistently downregulated (table 4).

Identification of genes differentially regulated in persistent wheeze

In the PW group, six individual samples were also compared with those from seven HC subjects and subsequently with the six previously mentioned TW samples. In these individual samples, the expression of 19 genes was $\geqslant$1.5-fold different

TABLE 2 Differentially expressed genes in heterogeneous wheezing phenotypes: genes downregulated relative to healthy control group

Gene	Symbol	GenBank#	Fold-change	
			TW	PW
Stress response-related genes				
MAPK/ERK kinase–ERK pathway				
Dual specificity phosphatase 1¶	*DUSP1*	NM_004417	-2.1	-2.0
Regulator of G-protein signalling 2, 24 kDa	*RGS2*	NM_002923	-2.1	-2.0
Dual specificity phosphatase 2¶	*DUSP2*	NM_004418	-1.5	-1.8
Jun B proto-oncogene¶	*JUNB*	NM_002229	-1.5	-1.5
Tumour necrosis factor (ligand) superfamily, member 13b¶	*TNFSF13B*	AF134715	-1.6	-1.6
Complement component 5 receptor 1 (C5a ligand)¶	*C5R1*	NM_001736	-3.2	-2.4
SNF1-like kinase	*SNF1LK*	NM_030751	-2.4	-2.1
cAMP responsive element modulator	*CREM*	AI800640	-2.0	-1.9
Other				
Ferritin, heavy polypeptide 1	*FTH1*	AA083483	-1.6	-1.5
Rho signalling pathway				
Protein tyrosine phosphatase type IVA, member 1	*PTP4A1*	AL578310	-1.5	-1.5
Nuclear receptor subfamily 4, group A, member 2	*NR4A2*	NM_006186	-3.1	-2.9
NF-κB pathway				
Tumour necrosis factor-α-induced protein 3¶	*TNFAIP3*	NM_00629	-1.5	-1.8
Growth arrest and DNA damage-inducible, alpha	*GADD45A*	NM_001924	-1.7	-1.7
Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 2¶	*LILRB2*	AF004231	-2.0	-2.0
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	*CDKN1A*	NM_000389	-1.8	-1.6
Leukotriene pathway				
Arachidonate 5-lipoxygenase	*ALOX5*	NM_000698	-1.5	-1.6
IFN-related				
IFN-inducible	*SLC7A7*	NM_003982	-2.0	-1.7
Other proliferation/cell death				
Transducer of ERBB2, 1	*TOB1*	BF240286	-2.1	-2.2
Hexokinase 3	*HK3*	NM_002115	-2.2	-1.7
Other genes				
C-type (calcium dependent, carbohydrate-recognition domain) lectin, super-family member 12	*CLECSF12*	AF313468	-2.2	-1.8
Mannosyl (α-1,3-)-glycoprotein β-1,2-*N*-acetylglucosaminyltransferase	*MGAT1*	N40551	-1.5	-1.6
Ring finger protein 157	*RNF157*	BC042501	-1.5	-1.5
TBC1 domain family, member 8 (with GRAM domain)	*TBC1D8*	NM_007063	-1.9	-1.5

TW: transient wheeze; PW: persistent wheeze; MAPK: mitogen-activated protein kinase; ERK: extracellular signal-regulated kinase; SNF: sucrose nonfermenting; cAMP: cyclic adenosine monosphosphate; NF-κB: nuclear factor-κB; TM: transmembrane; ITIM: immunoreceptor tyrosine-based inhibitory motif; IFN: interferon; ERBB2: v-erb-b2 erythroblastic leukemia viral oncogene homologue 2, neuro/glioblastoma derived oncogene homologue (avian); TBC: tre-2/USP6, BUB2 and cdc16; GRAM: glucosyltransferases, Rab-like GTPase activators and myotubularin. #: accession number; ¶: validated by semi-quantitative RT-PCR.

from that in HC subjects. In these samples, 13 genes were found to be upregulated, whereas six were downregulated. Among the upregulated genes, six were found to be immune-related, whereas the others were involved in protein folding and transportation. Among the downregulated genes, three were found to be immune-related. One of these genes was the prostaglandin (PG)E_2 receptor 2 (EP_2) gene (*PTGER2*) (table 5).

Validation by semi-quantitative RT-PCR

A total of six immune-related genes were validated, three differentially regulated in TW and three in PW. These genes were the annexin A1 gene (*ANXA1*), *STAT1* and the Toll-like receptor 7 (TLR7) gene (*TLR7*) in TW, and the genes encoding G-protein-coupled receptor 18 (*GPR18*) and granzyme H (*GZMH*) and *PTGER2* in PW. Semi-quantitative RT-PCR analysis confirmed the differential expression of these genes.

DISCUSSION

The present study is the first to apply microarray technology to CD4+ T-cells from wheezing infants in order to investigate whether or not gene expression profiles account for the heterogeneity in wheezing phenotypes. Furthermore, it was investigated whether these gene expression profiles might

TABLE 3 Differentially expressed genes in heterogeneous wheezing phenotypes: genes upregulated relative to healthy control group

Gene	Symbol	GenBank#	Fold-change	
			TW	PW
Stress response-related genes				
Heat shock proteins				
Heat shock 70 kDa protein 8	*HSPA8*	AB034951	1.5	1.6
Heat shock 90 kDa protein 1, β	*HSPCB*	AF275719	1.5	1.5
Heat shock 90 kDa protein 1, α	*HSPCA*	AI962933	1.7	1.7
Heat shock 70 kDa protein 1A¶	*HSPA1A*	NM_005345	1.5	1.5
Heat shock 70 kDa protein 4	*HSPA4*	AA043348	1.5	1.6
DnaJ (hsp40) homologue, subfamily A, member 1	*DNAJA1*	NM_001539	1.5	1.7
DnaJ (hsp40) homologue, subfamily C, member 3	*DNAJC3*	AL119957	1.5	1.5
MAPK/ERK kinase–ERK pathway				
Tumour necrosis factor receptor superfamily, member 25	*TNFRSF25*	U94510	1.6	1.6
Homeodomain-interacting protein kinase 1	*HIPK1*	AI393355	1.9	1.9
Mitogen-activated protein kinase kinase kinase kinase 5	*MAP4K5*	Z25426	1.4	1.6
Nuclear factor of activated T-cells 5, tonicity-responsive	*NFAT5*	NM_006599	1.4	1.5
Ras pathway				
Ras-GTPase-activating protein SH3-domain-binding protein	*G3BP*	NM_005754	1.5	1.5
Son of Sevenless homologue 1 (Drosophila)	*SOS1*	AW241962	1.7	1.7
Rab18, member Ras oncogene family	*RAB18*	AI769954	1.6	1.7
Ras p21 protein activator 2	*RASA2*	NM_006506	1.7	2.0
Rap2A, member of Ras oncogene family	*RAP2A*	AI302106	1.6	1.8
TGF-β signalling				
Janus kinase 1 (protein tyrosine kinase)	*JAK1*	AL555086	1.7	1.6
Protein kinase, lysine deficient 1	*PRKWNK1*	AI445745	1.5	1.5
NF-κB pathway				
A kinase (PRKA) anchor protein 13	*AKAP13*	AI674926	1.7	1.8
Butyrate-induced transcript 1	*HSPC121*	AJ271091	1.5	1.5
Other proliferation/cell death				
Phosphoinositide 3-kinase/Akt pathway				
Serine/threonine kinase 4	*STK4*	Z25430	1.7	1.8
Kinase interacting with leukaemia-associated gene (stathmin)	*KIS*	AW173222	1.7	1.9
Pre-B-cell leukaemia transcription factor interacting protein 1	*PBXIP1*	NM_020524	1.6	1.4
Other				
TP53-regulating kinase	*TP53RK*	BG339450	1.4	1.6
Apolipoprotein L, 1	*APOL1*	AF323540	1.7	1.5
Deoxyhypusine synthase	*DHPS*	NM_001930	1.5	1.5
Nuclear receptor-interacting protein 1(interacts with glucocorticoid receptor)	*NRIP1*	AI824012	1.9	2.4
Transducer of ERBB2, 2	*TOB2*	D64109	1.5	1.7
Ret finger protein 2, tumour suppressor gene	*RFP2*	BF939833	1.6	1.9
Transducin (β)-like 1 X-linked	*TBL1X*	AW968555	1.6	1.8
Dicer1, Dcr-1 homologue (Drosophila)	*DICER1*	NM_030621	1.6	1.6
Eukaryotic translation initiation factor 2C, 3	*EIF2C3*	NM_024852	1.5	1.5
Natural killer cells				
Natural killer cell receptor; natural killer-tumour recognition sequence	*NKTR*	AI688640	1.7	1.7
Natural killer cell receptor DNAM-1 (CD226)	*CD226*	NM_006566	1.4	1.5
Integrin αL (antigen CD11A (p180))	*ITGAL*	BC008777	1.6	1.7
Protein degradation				
CCR4-NOT transcription factor; proteasome; mRNA processing complex	*CNOT7*	NM_013354	1.6	1.6
Proteasome, component of cellular antioxidative system	*PSMB5*	BC004146	1.5	1.5
Hydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A thiolase	*HADHA*	BG472176	1.5	1.6
Cytokine-related				
Regulator of IL-2 expression	*ILF2*	NM_004515	1.5	1.5
IL-27 receptor, suppressive effect on T-cells, especially autoreactive Th17 cells	*IL27RA*	NM_004843	1.6	1.8
Cytokine-inducible SH2-containing protein (suppressor of cytokine signalling)	*CISH*	D83532	1.5	1.5

TABLE 3 continued

Gene	Symbol	GenBank#	Fold-change	
			TW	PW
Transport				
Nuclear protein transport factor	*NUP50*	AF267865	1.5	1.5
Leukocyte transport/binding				
Purine-rich element-binding protein A	*PURA*	NM_005859	1.6	1.6
Other genes				
Topoisomerase I binding, arginine/serine-rich	*TOPORS*	NM_005802	1.6	1.7
Phenylalanine-tRNA synthetase-like, α subunit	*FARSLA*	AD000092	1.5	1.5

TW: transient wheeze; PW: persistent wheeze; hsp40: heat shock protein 40; MAPK: mitogen-activated protein kinase; ERK: extracellular signal-regulated kinase; SH: Src homology; Rap: Ras-related protein; TGF-β: transforming growth factor-β; NF-κB: nuclear factor-κB; PRKA: adenosine 5′-monophosphate kinase-activated protein kinase; Akt: Akt kinase; TP53: tumour protein 53; ERBB2: v-erb-b2 erythroblastic leukemia viral oncogene homologue 2, neuro/glioblastoma derived oncogene homologue (avian); DNAM: DNAX accessory molecule; CCR: CC chemokine receptor; NOT: negative regulator of transcription; IL: interleukin; Th17: IL-17-producing T-helper 17 cell. #: accession number; ¶: validated by semi-quantitative RT-PCR.

contribute to the understanding of the immunopathology of wheezing. The present data establish that wheezing phenotypes share common gene expression profiles, but also show that the heterogeneity in wheezing phenotypes is, in part, reflected in the gene expression profiles of peripheral blood samples.

When comparing the common gene expression profiles of both types of wheezing infant, microarray analysis revealed differential gene expression of several stress response-related genes. For instance, decreased expression was found for *C5R1* of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway. Although C5 is a mediator of allergic reactions, an inverse association was seen between C5 and bronchial hyperresponsiveness. Interestingly, blocking *C5R1* inhibits the production of interleukin-12 by macrophages and monocytes. This cytokine drives type-I adaptive immune responses [16, 33]. Since it has become clear that part of the increase in symptoms of wheezing occurs due to not merely atopy but also an increase in bronchial hyperresponsiveness, *C5R1* might play a role in this phenomenon [3]. Increased expression was found of *HSPA1A*, a polymorphic variant of heat shock protein 70 (hsp70). In adult asthmatics, expression of hsp70 is increased in bronchoalveolar lavage samples in both epithelial cells and PBMCs, suggesting a role for hsp70 in asthma [34]. hsps are stress proteins, the expression of which is increased at sites of inflammation. Interestingly, the participants of the present study were only included when no symptoms of wheezing had been reported for $\geqslant$6 weeks (for the PW group) and no other episodes of fever had been reported. This may indicate that a parameter of inflammation continues to show increased expression even without recent symptoms of wheezing.

Decreased expression levels were also found for *JUNB*, *TNFAIP3*, *DUSP2*, *LILRB2* and *TNFSF13B*, all of which are involved in stress responses, *via* either the MAPK/ERK pathway or the nuclear factor-κB pathway. Of these genes, only *JUNB* has previously been associated with asthma, and has also been described as a Th2 transcription factor [35, 36]. This would imply that decreased expression of *JUNB*, as found in the present analysis, would be protective against allergen-induced airway inflammation. However, apart from the fact that this was a murine study, inclusion of the present participants was based purely on a history of wheezing, and not on atopic asthma. Interestingly, decreased expression of *TNFAIP3* and *DUSP2* has also been described in a microarray study of CD4+ T-cells in atopic dermatitis [21]. Taken together, the results of the present study suggest that CD4+ T-cells from infants with a reported history of wheezing, but without active disease, exhibit a prolonged activated state compared to controls.

In the TW infants, decreased expression of several IFN-related genes was found. Among these was *STAT1*. STAT1 is known to be the major intracellular response protein for both IFN-γ and IFN-α/β. In a murine study of HASHIMOTO *et al.* [37], it was shown that the absence of STAT1 resulted in airway dysfunction and an increase in airway mucus production following infection with RSV. During the acute phase of an RSV infection, suppressed production of IFN-γ from stimulated PBMCs was shown [38]. Subsequently, it was shown that reduced levels of IFN-γ in early life are associated with an increased risk of developing wheezing by the age of 1 yr [12]. In addition, decreased expression was found of TLR7. TLR7 is activated by single-stranded RNA viruses, such as RSV [39, 40]. In 2006, it was shown that the use of a TLR7 ligand could redirect allergen-specific Th2 responses, as well as allergen-induced hyperresponsiveness [41, 42]. These findings might provide a partial explanation for the subsequent wheezing following viral infections in TW.

In PW, differential expression of stress response-related genes was also found. Amongst others, decreased expression of EP_2 was found. The ligand for this receptor, PGE_2, is able to modulate the cytokine production of CD4+ T-cells towards a Th2 response [43, 44]. Furthermore, PGE_2 induces an increase in IgE production, and is, therefore, believed to be a mediator in the development of asthma [45]. A protective role of PGE_2 against bronchoconstriction has also been described. This would be an effect of PGE_2 on G-protein-coupled receptors, present on airway smooth muscle cells, and could explain why decreased expression of EP_2 would give rise to more bronchoconstriction [46]. However, whether or not the

TABLE 4 Differentially regulated genes[#] in six transient wheeze (TW) subjects

Gene	Symbol	GenBank[¶]	Fold-change	
			TW/HC	TW/PW
Stress response-related genes				
p53/Fas-ligand pathway				
Annexin A1; mediator of anti-inflammatory effect glucocorticoids[+]	*ANXA1*	NM_000700	1.5	1.5
Protein tyrosine phosphatase	*PTPN13*	NM_006264	1.3	1.5
MAPK/ERK kinase–ERK pathway				
AHNAK nucleoprotein (desmoyokin)	*AHNAK*	BG287862	1.6	1.5
Other				
Glutathione-*S*-transferase M1	*GSTM1*	X08020	1.9	1.8
Glutathione-*S*-transferase M1	*GSTM1*	NM_000848	2.0	1.8
Glutathione-*S*-transferase M1	*GSTM1*	NM_000561	2.2	1.9
Decapping enzyme hDcp2	*DCP2*	AI873425	-1.1	-1.5
IFN-related genes				
IFN-induced protein with tetratricopeptide repeats 2	*IFIT2*	AA131041	-2.0	-1.6
IFN-induced protein with tetratricopeptide repeats 3	*IFIT3*	AI075407	-5.5	-2.5
IFN-induced protein with tetratricopeptide repeats 3	*IFIT3*	NM_001549	-2.2	-1.4
IFN-inducible protein viperin	*cig5*	AW189843	-3.0	-2.0
Toll-like receptor 7, antiviral immunity[+]	*TLR7*	NM_016562	-1.6	-1.5
IFN-inducible transcription regulator[+]	*STAT1*	M97935_MB	-1.5	-1.5
Component of the IFN type III receptor	*IL28RA*	AW340139	-1.3	-1.5
Natural killer cells				
Natural killer cell receptor, IL-15 dependent; inhibitory immune receptor	*KLRB1*	NM_002258	1.8	2.0
Killer cell lectin-like receptor subfamily F, member 1	*KLRF1*	NM_016523	-2.6	-1.7
Phosphoinositide 3-kinase/Akt pathway				
SLAM family member 7	*SLAMF7*	AL121985	-1.9	-1.9
Insulin receptor	*INSR*	AA485908	-1.7	-1.7
NF-κB pathway				
B-cell RAG-associated protein	*GALNAC4S-6ST*	NM_014863	-2.2	-1.5
B-cell CLL/lymphoma 11A (zinc finger protein)	*BCL11A*	AI912275	-1.5	-1.5
B-cell CLL/lymphoma 11A (zinc finger protein)	*BCL11A*	NM_022893	-1.6	-1.5
HIV type I enhancer binding protein 3	*HIVEP3*	NM_024503	-1.6	-1.6
HIV type I enhancer binding protein 3	*HIVEP3*	AB046775	-1.5	0.9
Ras pathway				
Rab23, member Ras oncogene family	*RAB23*	AF161486	-1.3	-1.6
Other proliferation/cell death				
Ankyrin repeat domain 28	*ANKRD28*	N32051	1.6	1.6
SH3 multiple domains 4	*SH3MD4*	AL566989	-1.2	-1.5
Other				
Immunoglobulin heavy constant μ	*IGHM*	X17115	1.4	1.5
Tissue integrity/cytoskeleton	*EPPK1*	AL137725	-1.5	-1.5
CD63 activation marker/pulmonary type II cells	*LAMP3*	NM_014398	-2.9	-1.7
Transcription activator	*ZNF6*	AU157017	-1.3	-1.5
Cell membrane transporter (extracellular matrix of epithelial cells)	*SLC16A10*	N30257	-1.1	-1.6
Zinc finger protein 285	*ZNF285*	AW513227	-1.3	-1.5
GTP-binding protein 5 (putative)	*GTPBP5*	AI860690	-1.9	-1.7
mRNA expression:polymerase (RNA) II (DNA-directed) polypeptide D	*POLR2D*	BF432147	-1.3	-1.5

HC: healthy control; PW: persistent wheeze; MAPK: mitogen-activated protein kinase; ERK: extracellular signal-regulated kinase; AHNAK: giant (Hebrew); hDcp: human decapping enzyme; IFN: interferon; IL: interleukin; Akt: Akt kinase; SLAM: signalling lymphocyte activation molecule; NF-κB: nuclear factor-κB; RAG: recombination-activating gene; CLL: chronic lymphocytic leukaemia; SH: Src homology. [#]: showing a 1.5-fold change (increase or decrease) compared to the HC and/or PW group; [¶]: accession number; [+]: validated by semi-quantitative RT-PCR.

TABLE 5 Differentially regulated genes[#] in six persistent wheeze (PW) subjects

Gene	Symbol	GenBank[¶]	PW/HC	PW/TW
Stress response-related genes				
Rho signalling pathway				
Rho guanine nucleotide exchange factor 17	*ARHGEF17*	NM_014786	1.1	1.5
MAPK/ERK kinase–ERK pathway				
Scavenger receptor class B, member 1	*SCARB1*	AV708130	1.3	1.5
Other				
Trophoblast-derived noncoding RNA	*TncRNA*	AI042152	-1.4	-1.8
Prostaglandin E_2 receptor 2^+	*PTGER2*	NM_000956	-1.5	-1.6
RAR-related orphan receptor C	*RORC*	AI218580	-1.3	-1.5
TGF-β signalling				
SKI-like	*SKIL*	AW294869	1.6	1.5
Dachshund homologue 1 (Drosophila)	*DACH1*	AI650353	1.4	1.4
Chemokine-related				
G-protein-coupled receptor 18^+	*GPR18*	AF261135	1.9	1.5
G-protein-coupled receptor 114^+	*GPR114*	BF057784	1.1	1.5
Natural killer cells				
Granzyme H (cathepsin G-like 2, protein h-CCPX)$^+$	*GZMH*	M36118	3.0	4.6
v-yes-1 Yamaguchi sarcoma viral oncogene homologue 1	*YES1*	NM_005433	1.6	1.6
Other proliferation/cell death				
Plastin 3 (T-isoform)	*PLS3*	NM_005032	1.3	1.5
LIM and senescent cell antigen-like domains 3	*LIMS3*	AF288404	-1.9	-1.5
Cytochrome b_{561}	*CYB561*	AL514271	-1.3	-1.5
Other				
Acetyl coenzyme A/remodelling/endoplasmic reticulum	*ALCAT1*	AV717041	1.7	1.7
Peroxisomal biogenesis factor 12	*PEX12*	NM_000286	1.4	1.5
Chromosome 21 open reading frame 107	*C21orf107*	AJ002572	1.5	1.5
SH3 domain containing, Ysc84-like 1 (*Saccharomyces cerevisiae*)	*SH3YL1*	NM_015677	1.3	1.5
Unknown	*NY-REN-7*	AW514267	-1.5	-1.7

HC: healthy control; TW: transient wheeze; MAPK: mitogen-activated protein kinase; ERK: extracellular signal-regulated kinase; RAR: retinoic acid receptor; TGF-β: transforming growth factor-β; SKI: v-ski sarcoma viral oncogene homologue (avian); LIM: lens intrinsic membrane protein; SH: Src homology; Ysc84: protein involved in the organisation of the actin cytoskeleton. [#]: showing a 1.5-fold change (increase or decrease) compared to the HC and/or TW group; [¶]: accession number; [+]: validated by semi-quantitative RT-PCR.

expression of EP_2 on CD4+ T-cells is of influence on the effect of PGE_2 on smooth muscle cells in the lungs is not known.

One of the shortcomings of the present study may be the fact that the CD4+ T-cells used in this study are not the only cells playing a role in the pathogenesis of wheezing. Wheezing, and subsequently asthma, is a systemic disease involving several different cell types and tissues. The present objective was to seek a cell system which was readily obtainable and applicable for diagnostic purposes in the future. Using, for instance, epithelial cells might give different results [22]. Since gene expression in CD4+ T-cells can reflect several immune responses, patients were only included when free of active disease of any type, including during the 6 weeks before blood sampling. Furthermore, the present study did not reveal the increased expression of typical Th2 genes in CD4+ T-cells. This may support the hypothesis that even PW is not always an indication of atopic asthma, but might also be the result of increased lower airway inflammation [4]. This is further supported by findings in adult patients with atopic asthma, where genomic linkage analysis of a large population of patients did not reveal any Th2 involvement [15]. In addition, lack of Th2 involvement has been described by gene array analysis of CD4+ T-cells in atopic dermatitis, a disease exhibiting very high serum IgE levels [21].

Only relatively small numbers of patients were examined in the present study. As mentioned previously, the introduction of new techniques, such as mRNA amplification, into microarray studies has diminished the quantities of RNA required for microarray analysis. However, the use of peripheral blood from children still makes it difficult to obtain enough RNA to perform both microarray analysis and semi-quantitative RT-PCR validation. Patient numbers were further diminished by the stringent inclusion criteria used. This was required in order to obtain homogeneous phenotypes. Since no significant differences in expression profiles were found, the present authors are confident that the amounts used were sufficient and that differences in phenotyping were not present. In addition, because of the small sample sizes, it was necessary to select the genes for validation. It was decided to validate some immune-related genes. However, the present authors postulate that there are also interesting genes among the non-immune-related genes, such as *GSTM1* [47, 48]. Owing to the small

sample sizes, several potentially interesting subgroup analyses could not be performed, such as investigation into the influence of atopy in wheezing or the use of inhaled corticosteroids.

Importantly, differential gene expression levels were not examined in clinical resting states. This may imply that the gene profiles demonstrated are a reflection of the intrinsically different phenotypes of the CD4+ T-cells in the patient groups examined.

In conclusion, the data presented in the current study provide further insight into the genetic factors in CD4+ T-cells contributing to the heterogeneity in wheezing phenotypes and into the immunopathology underlying infant wheezing. Further research for the evaluation of the predictive quality of these gene expression profiles is currently being performed in a large group of prospectively included children.

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